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CYTOLOGICAL TECHNIQUE

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CYTOLOGICAL TECHNIQUE

BY

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DEDICATED WITH GRATITUDE
TO THE MEMORY OF
GILBERT C. BOURNE, F.R.S.
FORMERLY
PROFESSOR OF ZOOLOGY
AND
COMPARATIVE ANATOMY IN THE
UNIVERSITY OF OXFORD

'La science est une de ces grandes personnes, revêche, ardue et quinteuse, comme dit Montaigne, qu'il ne suffit nullement de désirer; il la faut gagner par beaucoup de soins, d'attention et de persévérance.'

Ces paroles sont applicable à toute science, mais elles le sont surtout aux sciences biologiques, à la cytologie en particulier.

J. B. Carnoy (1886 *b*.)

PREFACE

THIS book is founded on the experience gained in giving nine annual courses in cytological technique to advanced students in the Department of Zoology and Comparative Anatomy at Oxford. I want to express my gratitude to Professor E. S. Goodrich, F.R.S., in whose Department it has been my privilege to work since I was an undergraduate, and to Dr. H. M. Carleton, from whom I first began to learn cytology fourteen years ago. I cannot omit to mention my indebtedness to Mr. Frank Sherlock, whose manipulative skill in microtomy is well known to all Oxford zoologists.

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OXFORD

INTRODUCTION

THE title of this book would have to be inconveniently long if it were to be exact. In a book so small as this it is not possible to cover the whole field of cytology, and I have confined myself to the technique of making permanent preparations of animal cells. It is in no sense a histological book : the cell is regarded as a living entity, and not as a unit in a tissue. Histological methods for special tissues are not given. Nevertheless, the principles of cytological and histological technique are the same, and it is hoped that the book will serve also as an introduction to the methods of histology.

My guiding principle has been to give as few methods as possible, and to describe them in great detail, with full information as to the nature of the reagents used. It has been my object to tell the student throughout what he is doing, so far as is possible in such a difficult subject. All irrational methods, with which the literature abounds, have been carefully avoided. Many investigators have published formulae for fixatives and other fluids, without giving any indication of the researches which have led up to them. They have given no concrete evidence of why they are to be preferred to other fluids, nor of whether various proportions of the substances used were tried, with what results. Often they have mixed oxidizers and reducers, or acids and basic substances. In such cases one does not know to what extent the results obtained are due to the original components, and to what extent to the results of their reactions. One is working in the

dark, without knowing what one is doing. Such irrational methods often give satisfactory results, but one cannot properly interpret those results, and one cannot get better results than by the use of rational methods. Such considerations have led me to omit many well-known and much-used methods.

It is to be hoped that cytological technique will become a truly scientific subject, and that no one will think of publishing a new technique without having a full knowledge of the substances used, without considering the possibility of reactions between them, and without giving a full account of the results which caused him to choose certain substances and to mix them in certain proportions.

In reducing the number of methods to the absolute minimum, I have chosen those rational methods which have given the best results in my hands. Probably if I had worked with other tissues, I should have chosen other methods. I shall be well satisfied if the student really understands the few here described, for then he will have, I hope, a solid basis of understanding which will enable him to appreciate others already existing, and perhaps to invent new ones. I have written the practical instructions in the laboratory while actually engaged in doing the things described. I have hoped in this way to give a living account of the methods, and to include all the small details which one soon learns to perform almost without consciousness, and which one is very apt to forget to mention, and yet which often make all the difference between success and failure.

Limitation of space has prevented me from discussing colour tests for definite chemical substances contained in cells, despite the growing importance of this subject.

Technical methods are usually treated in an entirely impersonal way, but I have not hesitated to give biographical information. The cytologist is a human being, interested in other human beings, and especi-

ally in other cytologists. Nine hundred and ninety-nine people out of a thousand would have thought the effect of formaldehyde on the fingers merely a nuisance. F. Blum thought what it meant, and introduced one of the most important fixatives into cytological technique. Knowledge of the sort of way in which discoveries were made in the past may help towards the making of new discoveries.

In the future the study of the living cell will become ever more important, and the making of permanent preparations only accessory to it. I believe that it will always be an important accessory, and this book is intended to give both understanding and proficiency in it.

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CYTOLOGICAL TECHNIQUE

CHAPTER I

THE LIVING CELL

THE living cell of many-celled animals is very difficult to study, for several reasons. It is usually not possible to separate it from other cells without the help of various substances which kill it, and unless we separate it, we cannot get a good view of it. If we choose a cell which we can observe closely while still alive, we are still confronted with the difficulty that its contents are mostly colourless and transparent, and only distinguishable from one another, if at all, by relatively small differences in the degree to which they refract light. If we use all our patience as well as all our resources in ordinary and dark-ground illumination, we may see the structures shown in Fig. 1B. These structures may be seen while the cell still lies in the body-fluid of the animal from which it was taken, without the use of any fixative or stain.

The cell shown in the diagram is supposed to be an undifferentiated one. The cytoplasm is a homogeneous fluid, about four-fifths of which is water. Two classes of proteins, albumins and globulins, are in colloid solution in the water, as emulsoids ; that is, water molecules are contained in the molecular aggregates of protein. These two classes of proteins

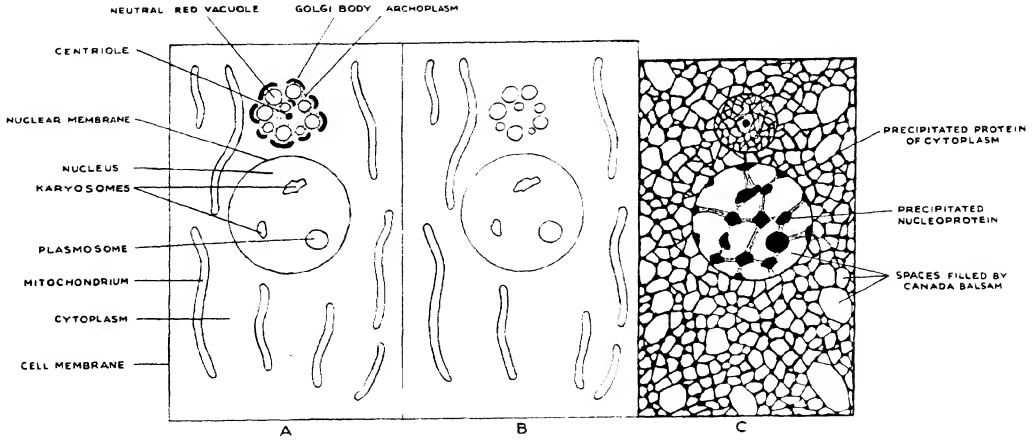


FIG. 1.—Diagram of an Undifferentiated Cell

A. Shows the cell as it probably exists during life
B. Shows what can be seen during life
C. Shows the cell fixed by a protein precipitant, sectioned and stained

both consist of many amino-acids, neither basic nor acidic ones preponderating. Both are, of course, soluble in weak salt solutions, like the water of the cell, but the globulins differ from albumins in being insoluble in distilled water. The aggregates of protein molecules are far too small to be seen, and we can only be made aware of their existence by the use of the ultramicroscope. They are far too small for any optical apparatus to produce an image of them, but the ultramicroscope will reveal diffraction haloes round them. Inorganic salts and sugar are in true solution in the water. The cytoplasm is bounded by a cell-membrane, which probably contains lipides. Floating in the cytoplasm are mitochondria, probably formed of phospholipides and protein in varying proportions in different cells. A group of vacuoles contains a watery fluid which stains easily with a dye called neutral red.

The nucleus is a large globule whose substance is separated from the cytoplasm by a membrane. This membrane is a morphological structure in the cell, and not simply an interface between two immiscible liquids. The nucleus consists of a watery fluid as homogeneous as the cytoplasm. It contains a special protein in colloid solution. This special protein is nucleoprotein or chromatin. The terminology of the nucleoproteins is unfortunately rather muddled. I shall follow Kossel (1918) in calling all compounds of nucleic acid with proteins nucleoproteins. Nucleic acid is the same substance in many and perhaps in all animal cells. It contains phosphoric acid, a carbohydrate, substances allied to uric acid and others. It is probably hexavalent (Mathews, 1930) and capable of combining with various amounts of various proteins. It appears that in most nuclei the combination between nucleic acid and proteins is not simply that of a salt, though Osborne (1924) thinks that plant nucleoproteins may always be salts. When the nucleoprotein

is not obviously a salt, and the protein or proteins concerned not particularly basic, I shall call the nucleoprotein a nuclein. A part of the protein may often be split off from a nuclein by peptic digestion, while the rest resists peptic digestion but can be separated from the nucleic acid by trypsin. (Many authors reserve the word nuclein for the substance left after peptic digestion, but this appears to be a somewhat artificial nomenclature.) There is another type of nucleoprotein, in which the combination is simply that of a salt, the acid radicle being nucleic acid and the basic radicle a basic protein, either protamine or histone. The nucleoprotamines, which constitute nearly the whole of the head of the sperms of the salmon and certain other fishes, are examples of this type of nucleoproteins. Protamines are very simple proteins, consisting of few amino-acids. They are strongly basic, and so is their compound with nucleic acid. The nucleohistones are similar to the nucleoprotamines, but histones are not so strongly basic, and they consist of more amino-acids. They occur in the nuclei of many cells. The nucleoprotein of thymus gland, which is an easy one to extract, is mostly nucleohistone, but nuclein is also present. We shall study the effect of fixatives on this mixture of nucleohistone and nuclein later in this book. Nucleohistone occurs also in the nuclei of birds' blood-corpuscles, and in sea-urchin sperms. It is evidently a widely distributed component of nuclei. Ordinary nuclei contain nuclein and often nucleohistone. Nuclein is an acidic substance, because the protein component is not strongly basic, while nucleic acid is quite a strong acid. Accordingly the nucleoprotein of most nuclei is acid, though any great admixture of nucleohistone would make it less so. It could become yet more so by the incorporation of more nucleic acid in the molecule, or by the loss of basic amino-acids from the protein component. Something of this sort must occur in the prophase of

cell division, for the chromosomes, when they appear, become more and more acidic, and therefore stain more and more strongly with the basic dyes. Their intense avidity for basic dyes is the reason for their name, which means colour-bodies. In the fully formed chromosomes the nucleic acid may perhaps be present as such (Mathews, 1930), and not in combination with protein at all. When, in telophase, the chromosomes disappear, basic proteins are apparently incorporated in the molecule, which goes into colloid solution.

In addition to the dissolved nucleoprotein, there are usually a few lumps in the nucleus, called karyosomes. These may be shown by microchemical tests to contain nucleic acid. In addition there is often a protein sphere called a plasmosome, which does not contain nucleic acid, and whose significance is obscure.

There is very strong evidence from genetics that the chromosomes do not lose their continuity between one cell division and the next. The cytological evidence is that in the great majority of cases (though not all) the nucleic acid which has largely constituted the chromosomes during cell division is mostly dissolved between one cell division and the next, in the form of nucleoprotein. This paradoxical disagreement between genetics and cytology provides one of the major problems of biology. The fact that in a few cases the chromosomes can be followed from one cell division to the next does not resolve the difficulty that in the great majority of cases the substance of the chromosome is, so far as one can see, dissolved. Those who have attempted to resolve the problem have mostly stultified their work by using fixatives which precipitate nucleoprotein in an unlifelike way. Perhaps there are invisible chains of genes in the nucleus, and nucleic acid only attaches itself to these during cell division.

The structures already mentioned are all that can be seen in the living cell in most cases, but there are

other structures which may be regarded with some certainty as existing, because the most diverse treatments bring them into evidence. These are shown in Fig. 1A, which represents an undifferentiated cell with all the structures which it probably contains during life. Those which we have not seen during the life of the cell are the centriole, archoplasm, and Golgi bodies. The first two are occasionally seen during life (Heiderich, 1910), the last extremely rarely, but there are sound reasons for believing in its existence. The centriole is a granule which divides into two at cell division and appears to play an important part in that process. Its chemical composition is unknown. Striations called asters radiate in the cytoplasm from the centrioles during cell division, and a spindle-shaped mass of cytoplasm stretches between them. Most fixatives produce striation in the spindle, but the observational and experimental evidence is that it is homogeneous until treated with a protein precipitant. Once again there is a conflict with genetics, for geneticists find peculiarities at that part of the chromosome which appears, in fixed cells, to be attached to a spindle fibre.

The protoplasm round the centriole is often denser than the cytoplasm as a whole, and is called the archoplasm. The neutral red vacuoles often lie in it. Round it are distributed the Golgi bodies, often banana-shaped. These probably consist of a lipide, but the evidence is not conclusive.

I have figured an undifferentiated cell. It remains to add that the mitochondria might have been drawn as granules, the centriole as double, the neutral red vacuoles as separate from the archoplasm, the archoplasm and the Golgi element as dispersed through the cytoplasm, and the karysomes many or absent. Protein granules might have been drawn in the cytoplasm. The cytoplasm might have been a gel and not a sol. Further, it must be realized that the particular functions of the cell in various parts of the

body will require the greatest diversity of shape and structure. The parts drawn and described are those which are of nearly universal occurrence.

The study of the living cell will become of supreme importance when someone has thought of a convenient way of examining it. Till then, and probably always, we must rely partly on preserving the cell in a fairly life-like condition, cutting thin sections of it, and staining them differentially so as to show up clearly the previously transparent parts. The advantages of so doing are so obvious as not to require discussion. Unfortunately we generally make the cell most unlife-like when we make permanent preparations of it in this way. (See Fig. 1c.) The whole cell is shrunken. The cytoplasm, previously homogeneous, is usually precipitated in the form of a spongework. The mitochondria, Golgi bodies and neutral red vacuoles are dissolved away. The nucleoprotein is precipitated in the form of a coarse spongework, holding particles at the junctions of the threads of the sponge. It is the purpose of this book to describe methods of preserving cells or parts of cells in a life-like way, and to explain them, so far as is possible with our present knowledge, in terms of chemistry. This is the process usually adopted. A small piece of tissue is soaked in a solution which prevents decay and renders the contents of the cells insoluble. This is called fixation. The fixative must usually be washed out. Then it must be impregnated throughout with melted paraffin. This can only be done indirectly. The water must be completely extracted with alcohol. Paraffin and alcohol will not mix, so the alcohol must be extracted with a substance which mixes with both. Benzene and cedarwood oil are convenient for this purpose. Then the tissue must be soaked in changes of paraffin until the benzene or cedarwood oil is extracted. The melted paraffin is then cooled to a solid block. This is cut into very thin sections with a slicer called a micro-

tome. The sections, still impregnated with paraffin, are attached to a glass slide. The paraffin is dissolved away, usually with xylene, and this is washed away with pure alcohol. This is replaced by weak alcohol or water, and the section is then immersed in a solution of a stain. After staining the section is passed through alcohol to xylene once more. A drop of a resin called Canada balsam dissolved in xylene is put on a coverslip, and this is lowered on to the section. The slide is kept on a warm plate until the xylene has mostly evaporated away. The balsam is transparent and has the same refractive index as glass. It dries hard and holds the coverslip firmly in position. If everything has been done carefully and intelligently, the cell should be seen with little distortion, the parts showing up clearly in one or more colours ; and the maker of the slide should have some concept of what he has done.

CHAPTER II

METHODS OF RESEARCH ON FIXATION

IF we cut a piece of tissue out of an animal and leave it without further treatment, it will soon become very unlife-like. It will dry up and shrink unless we keep it wet. If we keep it wet with a salt solution of the proper osmotic pressure, it will not immediately undergo any large changes, but soon bacteria will begin to multiply and destroy the tissue unless we prevent them. Even if we keep out bacteria by strict asepsis, changes will occur. Every cell contains enzymes, which synthesize the amino-acids brought to it into the particular proteins of the cell. After death the cell becomes acid, and when this happens these enzymes begin to work in the wrong direction, splitting the proteins into amino-acids, which diffuse away out of the cell. This sort of decay is called autolysis. If we prevented this from happening, should we have 'fixed' the cell? We should not, for we have not only to prevent ordinary post-mortem changes in the cell, but also to prevent subsequent necessary treatment from causing unwanted changes. We are going to cut thin sections of cells, and the substances in them would dissolve out later unless they were rendered insoluble. They would also shrink and become distorted when put in such unfavourable fluids as hot melted paraffin. The substances of the cell must be made insoluble, then, and also prevented from subsequent shrinkage. To sum up, cytological fixatives are solutions which prevent bacterial decay

and autolysis, and render the substances of the cell insoluble and prevent its subsequent shrinkage and distortion. These are not the only effects of fixatives. They often change the refractive indices of various parts of the cell and thus make them more easily seen. This is not usually very helpful, for the parts will generally be stained after fixation; but a very useful property of fixatives is that they usually make the parts of the cell easily stainable.

The substances which appear to me to be important as fixatives are the following: ethyl alcohol, formaldehyde, acetic acid, picric acid, chromic acid, potassium dichromate, mercuric chloride, cobalt nitrate, and osmium tetroxide. A large number of substances has been used, but I doubt whether better results can be obtained than by a judicious use of these nine. Each has its advantages and disadvantages, and fixing fluids are therefore usually mixtures of two or more. People with no chemical knowledge have often mixed them together in quite a haphazard way, so that their fixative properties have been destroyed. The total number of useless fixatives with names attached to them is very great. In this book I describe only six fixing mixtures, invented by men who knew what they were doing, and whose names deserve to be perpetuated. We cannot begin to understand mixtures, however, until we have first studied the properties of their components. Let us see how these properties have been studied. The chief properties which we must consider are (1) their effects on proteins, lipides, and carbohydrates, as revealed by precipitation and other experiments; (2) the artificial appearances which they may cause in cells; (3) the rate at which they penetrate; (4) the degree to which they alter the volume of cells, and protect cells from subsequent shrinkage; and (5) their effects in altering the physical properties of tissues.

In this chapter we shall only study the methods

of attack of those who have made a general study of some aspect of fixation. In the next chapter we shall study their results, and also the results of those who have made special investigations of the separate substances. The next chapter will naturally be the more interesting, but the methods by which the knowledge was obtained must not be neglected.

(1) *Precipitation experiments.* Proteins are dispersed throughout the cell, and therefore they are more important than lipides and carbohydrates in its general fixation. We shall only study the chemistry of the action of fixatives on proteins here, leaving the lipides and carbohydrates to the part of the book where the various fixatives are described separately.

The first man to put the subject of fixation on a really scientific basis was Alfred Fischer, who described his results in his great book, *Fixierung, Färbung und Bau des Protoplasmas*, in 1899. Fischer mixed protein solutions with fixatives in small bottles or in Petri dishes, and left them for a day. He noted whether precipitates were formed, and if so what they were like, and whether they were soluble in water. He studied many proteins and meta-proteins, but the only ones which are of real interest to the cytologist are albumin (from blood serum and from egg-white), globulin (from blood serum), nuclein (from yeast), and nucleic acid (from yeast and from thymus gland). (He also studied what he called ' nucleo-albumin ', and his results with this have been quoted in more than one book as though the substance were nucleoprotein. Reference to his own book will show that he was studying casein, a protein which contains no nucleic acid whatever, though it contains phosphorus. Casein is of very little significance to the cytologist.) He usually used a 2% solution of protein. He dissolved albumin in water, and globulin and nuclein in warm 0·2% potassium hydroxide. The yeast nucleic acid he dissolved in

warm water, the thymus nucleic acid in 2% potassium hydroxide. It appears, from indirect evidence, that he used only small amounts of the fixatives in comparison with the amounts of the proteins. This was unfortunate, for in actual practice the fixative is generally present in great excess. The result sometimes differs according to whether fixative or protein is in excess.

Mann (1902) extended the work of Fischer by means of the test-tube experiments, chiefly with egg-albumin, and Berg made experiments with nuclein and nucleic acid (1903) and with nucleoprotamines (1905). This work on the nucleins and nucleic acid is in the main confirmatory of Fischer, but some of his results were different. Both Fischer and Berg used commercial vegetable nucleins. I am not aware that any one has previously studied the effects of fixatives on animal nucleoproteins (though they have studied nucleic acid from animal sources). I have therefore made a study of the mixed nucleoproteins (nucleohistone and nuclein) of thymus gland, that is, chiefly the nucleoproteins of the interkinetic nuclei of white blood-corpuscles. In the preparation of nucleoproteins, alcohol is usually used to wash the product. Since it is probable that alcohol has a chemical as well as an obvious physical effect on ordinary proteins, it seems best to avoid its use when studying nucleoproteins, if we are to study them so far as possible as they occur in the living cell. I therefore prepared a solution of nucleoprotein as follows :

The fresh thymus of a calf was freed from fat. 100 grams of it was minced with a machine, ground up with clean sand, and extracted for a day with a litre of distilled water, a few drops of chloroform being added to prevent decay. The fluid was then filtered, which took a day. 20 c.c. of 10% acetic acid was added to the filtrate, which precipitated the nucleoproteins. The precipitate was filtered off

and washed twice with a litre of $\frac{1}{4}\%$ acetic acid. It was then dissolved in a litre of $\frac{1}{2}\%$ potassium hydroxide. It took about a day to dissolve. A small residue remained undissolved. This was separated by filtration and discarded. The solution was now an alkaline solution of nucleoprotein. This was brought as close to neutrality as was possible without danger of precipitation, by the addition of 4·6 c.c. of 10% acetic acid to every 100 c.c. of nucleoprotein solution. 100 c.c. of the solution were evaporated to dryness, and found to contain 0·725 gram of nucleoprotein. 69 c.c. of the solution were measured out and made up to 100 c.c. with distilled water. The solution was now approximately a $\frac{1}{2}\%$ solution of nucleoproteins in weak potassium acetate solution. This was the nucleoprotein solution used in the experiments described later in this book.

To make an albumin solution for a series of experiments comparable in general with those of Fischer and Mann, some egg-white was measured out and four times its volume of distilled water added and the mixture beaten up. The globulin was precipitated by the distilled water. The fluid was then filtered, which is a very slow process. The filtrate was roughly a 2% solution of albumin. (A crystal of thymol may be added to such solutions to prevent rapid decay.) The experiments with nucleoprotein and albumin solutions, described in the next chapter, are very easy to carry out. Precise instructions are given. The results will be as stated if they are carefully followed.

(2) *Artefacts.* In the last year of last century there was published a paper in the *Journal of Physiology*, which must rank along with Flemming's 'Mittheilungen zur Färbetechnik' (1884), Heidenhain's 'Über Kern und Protoplasma' (1892), Fischer's 'Fixierung, Färbung und Bau des Protoplasmas' (1899), Tellyesniczky's 'Über die Fixierungs—(Härtungs—) Flüssigkeiten' (1898) and Mann's

' Physiological Histology ' (1902), as one of the classics of cytological technique. The paper referred to is Hardy's ' On the Structure of Cell Protoplasm ' (1899). Previously it had been supposed that the spongework seen in sections of fixed material (Fig. 1c) represented a structure that was present in the living cell. People thought that there was a spongework of denser cytoplasm holding a less dense cytoplasm in its meshes. Hardy made a solution of egg-albumin, took drops of it in loops of silk thread, and fixed them in various fixatives. He also put larger drops in moulds of porous paper and fixed them. He cut thin sections of fixed albumin solution and stained them with iron haematoxylin. (See p. 99.) He showed that the albumin was precipitated as a spongework, looking like a network in thin sections. In thicker sections a haze appears to fill the meshes. This is simply caused by the strands of the spongework, out of focus above and below the spaces. In extremely thin sections (0·6–1·0 μ thick), no substance whatever can be demonstrated between the strands, not even with undifferentiated iron haematoxylin. The space between is simply filled with Canada balsam or other mounting medium. The size of the meshes, both with albumin solutions and with the cytoplasm of cells, depends on the fixative used. Some fixatives produce a very coarse mesh, others a very fine one. Spherical masses occur at the points where one strand meets another. The whole spongework is an artefact. The cytoplasm of cells, like an albumin solution, is a colloid solution of protein in water.

Fischer (1899) impregnated elder-pith with protein solutions under reduced pressure, fixed in various ways, sectioned the pith and obtained somewhat similar results independently of Hardy.

What Hardy did for the cytoplasm, Tellyesniczky did for the nucleus (1902 and 1905). He showed that the ground substance of the nucleus is homo-

geneous. His reasons for disbelieving in the meshwork in the nucleus were (1) that it is invisible during life; (2) it does not appear after fixation with those fixatives that are not protein precipitants; (3) that it does appear after fixation with protein precipitants; and (4) that in appearance it resembles a protein coagulum. His observations were chiefly on the large spermatogonia of the salamander. In the nucleus is suspended a plasmosome and often several karyosomes. In a few nuclei, for example those of the lung and mesentery cells of the newt, the living nucleus is cloudy, with irregular spots and stripes; but this is most unusual. It must be mentioned that so recently as 1927 (Martens) the presence of a network in the living nucleus has been upheld, in the case of the young ovules of orchids and in cells of the styles of several grasses. There seems little doubt, however, that the ground substance of the nucleus is structureless, and that the network or spongework is an artefact produced by fixation. Still tradition dies hard, and those fixatives which produce a clear, sharply defined net are often spoken of as 'good' nuclear fixatives, while those which preserve the life-like condition are called 'bad'.

Before Tellyesniczky had studied the living nucleus in detail, he had studied the effects of various fixatives on the large spermatogonia and primary spermatocytes of the salamander, and had published his results in his important paper, 'Über die Fixierungs—(Härtungs—) Flüssigkeiten' (1898). He chose these cells because they are large and spherical, contain no secretory products, are undifferentiated, and are very susceptible to the effects of reagents. After fixation he embedded and sectioned the material, and made a careful study of the result with each fixative.

Strangeways and Canti (1927) studied the living cell and watched it being fixed, using dark-ground

illumination in order to overcome its transparency. Their paper is illustrated by beautiful figures, which deserve careful study. They worked with cells from tissue-cultures of the choroid and sclerotic of the chick embryo. They found no structure in the ground cytoplasm or ground nucleoplasm before fixation. Their work is a valuable contribution to the investigation of the immediate effects of fixatives, but of course does not teach us anything of the degree to which fixatives protect the cell against the reagents in which it is soaked after fixation, which is one of the most important functions of fixatives. Thus the work is complementary to that of Tellyesniczky, who did not study the immediate but only the remote effects.

(3) *Rate of Penetration.* Fixing fluids diffuse into cells, but the process is more complicated than simple diffusion of indifferent substances into protein solutions, because fixing fluids fix as they go, and the fixed proteins are in varying degrees impermeable to the fixative. Thus fixatives make a barrier against their own penetration, unless they are without effect upon albumins and globulins, as acetic acid is. Acetic acid penetrates more rapidly than any other fixative, simply because it does not form a barrier against itself. It dashes through the tissues, fixing the nucleoproteins, but leaving the cytoplasm unfixed. Tellyesneczky (1927) carefully poured fixatives on to the surface of white of egg, in such a way as to avoid mixing of the fluids, and noticed that a membrane was at once formed, which protected the rest of the albumin solution from precipitation. The membrane gradually grew in thickness, as the fixative penetrated through it and fixed the underlying albumin. He measured the thickness of the membrane after three days with various fixatives. With tannin and platinum chloride, both of which are powerful precipitants of proteins in test tube experiments, the membrane remained immeasurably thin throughout this time. No penetra-

tion whatever took place, while saturated mercuric chloride penetrated 10 mm. or more. Tellyesniczky concludes that tannin and platinum chloride are useless as fixatives, though the latter has been largely used, despite its high cost.

Tellyesniczky also put pieces of liver, spleen, kidney and brain into fixatives, and cut them open at various periods thereafter to note how far they had penetrated, by naked-eye observation of visible changes in the fixed part. Once again he found tannin and platinum chloride the slowest.

Tellyesniczky found that 2% osmium tetroxide penetrated about twice as quickly as 1%. The ordinary rate of diffusion is increased about 2% for each degree Centigrade that the temperature is increased, but Tellyesniczky found that in fixation this figure is not approached. Since decay and maceration occur much more quickly at higher temperatures, he does not recommend fixing at the body temperature of warm-blooded animals. He found that in mixtures one of the components often penetrated more rapidly than another.

Miss Underhill (1932), working under my supervision, studied the rate of penetration of fixatives into cylindrical masses of cavy liver, made with a cork-borer of internal diameter 0·7 mm. Liver was chosen for the study as being more homogeneous than most tissues, and firm enough to give good borings. The fixatives were generally allowed to act for a quarter of an hour, and the pieces were then left for a week in a macerating fluid, to make the distinction between fixed and unfixed cells as sharp as possible. The tissue was embedded, sectioned transversely, and stained. The amount of penetration was measured under the microscope by means of a micrometer eye-piece. Seven to eleven cylinders of tissue were used in the study of the action of each fixative, and means were calculated. Tellyesniczky's figures, on the contrary, appear to have been based on one measurement each.

(4) *Shrinkage and Swelling.* Perfectly even alteration in volume would not be very harmful, but shrinkage caused by fixatives is always unequal in different parts and this distorts the cell. Kaiserling and Germer (1893) took eggs from the ovaries of cows and measured them in the follicular liquid. Four diameters, at 45° from one another, were measured, and the mean taken. They were then washed free from the follicular liquid with salt solution, so that the proteins of the follicular liquid should not be precipitated later and so obscure the eggs. The eggs were measured as before, and then transferred to various fixatives. After about a quarter of an hour's fixation they were measured again. Unfortunately the salt solution used was hypotonic (0·6–0·75%), and the eggs naturally swelled in it.

Berg (quoted by Tellyesniczky, 1927) measured the volume of livers and spleens before and after fixation, and after the various other processes up to embedding, with the various fixatives. Patten and Philpott (1922) studied the shrinkage and swelling caused by fixation in pig embryos. The crown-rump length was first measured to the nearest $\frac{1}{10}$ mm., and after fixation and other treatments up to melted paraffin it was measured again. Unfortunately the only pure substance tested was formaldehyde, for the author's object was to be able to compare the amount of change in size caused by the usual fixing mixtures. Tarkhan (1931), working under Carleton's supervision, made an ingenious study of shrinkage. He attached two hooks to the ends of a piece of tissue. One piece was secured near the bottom of an upright tube. The piece of tissue was arranged with its long axis vertical. The hook fixed into its upper end was connected by a thread to one end of a lever, whose other end made a mark on blacked paper on a very slowly revolving drum. The mark gave a permanent record of the shrinkage caused by fixation and by the subsequent processes. The results obtained were however only

relative. Absolute measurements were unfortunately not recorded. Liver, spleen, intestine and limb-muscles of the cat and rabbit were studied. Seventy fixations were made. Thirty were carried right through to the melted paraffin, a hot-water jacket being placed round the tube for this purpose.

It might be thought that the osmotic pressure of fixatives would influence the shrinkage or swelling that they cause, and people have suggested that they should be isotonic with the cell. It is obvious that when fairly strong solutions are used, it can only make them a little more hypertonic if one dissolves them in 0·9% saline than if one dissolves them in distilled water. It is an extraordinary fact that the inventors of fixing fluids have sometimes been fascinated by physiological saline, as though this could in itself in some miraculous way make solutions of other salts in it isotonic. Similarly, fixatives for marine animals are often made up in sea water instead of distilled water, as though that made them isotonic with the tissues.

Tellyesniczky (1927) is of opinion that the osmotic pressure of a fixative is in itself of no significance, although the concentration of its constituents will be of importance in other ways, in determining the hardness or softness of the protein precipitates, the degree of preservation of lipides, and the rate of penetration. He thinks that when a cell begins to be fixed, it is changed in such a way that it no longer responds to changes in osmotic pressure.

Mr. C. E. J. Crawford and I (unpublished results) have thought it worth while to test the osmotic pressures of fixatives by freezing-point determinations, despite Tellyesniczky's very reasonable criticism. Two of the fixatives, picric acid and mercuric chloride, presented the difficulty that they are usually used in saturated solution. We could only determine the osmotic pressure of a saturated solution in an indirect manner, because cooling to freezing-point naturally

results in precipitation, and hence weakening of the solution, and hence lowering of the osmotic pressure. We have therefore used half-saturated solutions and doubled the osmotic pressures obtained. The results are necessarily only approximate. It will be realized that any fixative consisting of or containing a saturated solution will vary slightly in concentration (and hence osmotic pressure) according to the temperature of the room at the time when the solution was made up. We have tested fixing mixtures as well as the unmixed substances, because in mixtures each constituent may alter the degree of dissociation, and therefore the osmotic pressure, of the others. One could not calculate the osmotic pressures of mixtures from a knowledge of those of their constituents. Bouin's fluid (p. 65) has such a high osmotic pressure that our thermometer would not measure it. We therefore got an approximate figure by mixing the fluid with an equal volume of water and doubling the osmotic pressure obtained.

(5) *The Physical Properties of Fixed Tissues.* Some fixatives make tissues hard, others leave them soft. If they are hard, they may be tough or brittle. It would be valuable to have numerical data expressing the physical properties of tissues after fixation by the different fixatives, for the ease of sectioning depends upon them. So far we have little exact knowledge, and must rely mainly on the general impressions that every one gains who does any cytological or histological work. Wetzel (1920), however, has investigated the elasticity of tissues after fixation, thinking that elasticity determines not only the behaviour of the tissue under the razor, but also its resistance to the disturbing effect of the diffusion currents which cannot be avoided when it is transferred from one fluid to another. He used the belly muscle of the cat for his observations. An elongated piece was cut out and fixed. The piece was then attached to a vertical support by one end, in such a way that it projected

horizontally. A weight was attached to the free end, and the amount of sag carefully measured. The tissue was kept wet during the process. The elasticity was calculated from a formula involving the dimensions of the piece of tissue, the weight, and the amount of sag. The higher the figure of elasticity, the more difficult is the tissue to bend. The figure with the different fixatives varied from about 4,500 with alcohol to only 9 with acetic acid. The figure for alcohol is about one-sixth of that for pine-wood. It appears that Wetzel made only one or two measurements with each fixing fluid. When two measurements were made, large discrepancies were found in the results, probably depending on the relative amounts of muscle and connective tissue in the piece tested. Wetzel calculated his results to a large number of figures, which was shown to be quite unjustified by the great discrepancies. It would have been far better to have calculated his results to only two significant figures, but to have made numerous observations with each fixative. Nevertheless, Wetzel's work is a start along an important line.

We have discussed the methods of those who have studied the physical effects of fixatives on cells and parts of cells. We have not discussed the chemistry of fixation, since the different fixatives act so differently, from a chemical point of view, that each substance is best treated separately. The little that is known on the subject is reserved for the next chapter.

It is generally best to kill animals for cytological work by a blow on the head or by cutting off the head. The piece of tissue, usually about 2 mm. thick, should be cut out directly after death with as little roughness as possible. It should not be held in forceps, but transferred with a scalpel. A smart tap on the scalpel will shake it into the fixative. Immersion of the scalpel in certain fixatives causes chemical interaction and is to be avoided.

CHAPTER III

SIMPLE FIXATIVES

Ethyl Alcohol

Suitable concentration for fixation : undiluted

ETHYL alcohol, C_2H_5OH, is a colourless liquid, miscible with water in all proportions. It was introduced as an anatomical preservative by Robert Boyle in 1663. The discovery was made at Oxford (Gunther, 1925). Alcohol is prepared from starchy substances, such as potatoes and grain, which are mashed up with water. Germinating barley grain is added. This contains an enzyme which changes the starch into sugars in less than an hour. Yeast is now added, which converts the sugars into alcohol and carbon dioxide. Strong alcohol is obtained from this weak solution by distillation at 78° C. All except the last traces of water are removed from the distillate by quicklime. Commercial absolute alcohol contains less than $\frac{1}{2}\%$ of water.

Alcohol is a reducing agent, being easily oxidized to acetaldehyde and thence to acetic acid. It must therefore not be used in mixtures with chromic acid, potassium dichromate or osmium tetroxide.

The following experiments show the effects of alcohol on protein solutions :

Place 1 c.c. of the albumin solution described in the last chapter in a test tube and add 9 c.c. of absolute alcohol. A light flaky precipitate is formed instantaneously. When this has settled remove the

supernatant fluid with a pipette, and add 10 c.c. of distilled water. The precipitate is insoluble. Add 2 c.c. of a saturated solution of sodium chloride. The precipitate does not dissolve. This distinguishes the precipitate from that formed by mercuric chloride.

Repeat the experiment using the nucleoprotein solution, prepared as directed in the last chapter, instead of albumin. There is now no immediate precipitate, but it has formed in a quarter of an hour and gradually falls to the bottom. Remove the fluid and add 10 c.c. of distilled water. The precipitate dissolves instantaneously, in striking contrast to the albumin precipitate. It is clear that alcohol is not a fixative for chromatin.

Fischer found that albumin, globulin and nucleic acid were precipitated by alcohol, the two former giving insoluble and the latter soluble precipitates. Berg found nuclein precipitates sometimes soluble, sometimes insoluble.

The rendering insoluble of proteins by alcohols, strong acids and salts of heavy metals is called 'denaturation'. (See under mercuric chloride.) It appears (Jordan Lloyd, 1926) that denaturation by alcohol may be a different process from denaturation by strong acids and heavy metals, which, as we shall see, is a similar process to coagulation by heat. (See under mercuric chloride.) In all cases the linkages of the proteins appear to be altered. Not only is solubility affected, but also the combining capacity with both acids and bases is increased, and susceptibility to enzymes changed. The change of combining capacity makes for easier staining, but it is less marked with alcohol than with other fixatives.

Hardy and Gardiner (1910) showed that the albumins and globulins of horse blood-plasma, precipitated by alcohol at freezing-point or below, are freely soluble in water. This shows that alcoholic fixatives should not be used at low temperatures.

Fats and phospholipides are soluble in alcohol,

which must obviously be avoided in fixatives when it is desired to preserve them. In general, therefore, alcohol is to be avoided in studies of the Golgi bodies and mitochondria, though those mitochondria that contain a large protein component are not dissolved.

Glycogen is precipitated by alcohol, but in no sense fixed, for it retains its solubility in water. It is used when glycogen is to be studied, but fluids containing less than 50% alcohol are to be avoided in the after-treatment.

Underhill found alcohol the third fastest penetrator of the fixatives she tried, being exceeded by acetic acid and mercuric chloride. It penetrated 0·46 mm. in a quarter of an hour. Tellyesniczky found that 90% alcohol penetrated moderately rapidly, 3½ mm. in twelve hours. Short fixation (one to a few hours) suffices for small pieces.

Kaiserling and Germer found that it shrunk cows' eggs greatly and irregularly, e.g. from 137 μ in diameter in saline to 101 μ.

Berg found reduction of 18% in volume of liver after fixation in 96% alcohol. This is a large amount, but less than with chromic and picric acids.

Wetzel's figure for resistance to bending is 4,500, much more than with any other fixative.

Alcohol naturally requires no special washing out. Tissues may be transferred directly to benzene or cedarwood oil and embedded. It leaves the tissues in a less stainable state than any other fixative. Only haematoxylin can be relied upon to stain after alcohol fixation.

Fig. 2 represents stained sections of spermatogonia of the salamander, fixed in various ways. The living cell is also shown, for comparison. The figures are taken from papers by Tellyesniczky (1898 and 1905). The cell fixed in alcohol is very characteristic. As alcohol diffuses into tissues, it shrinks the cytoplasm of the cells as it precipitates it, and carries it before it until the precipitate is held back by the cell-mem-

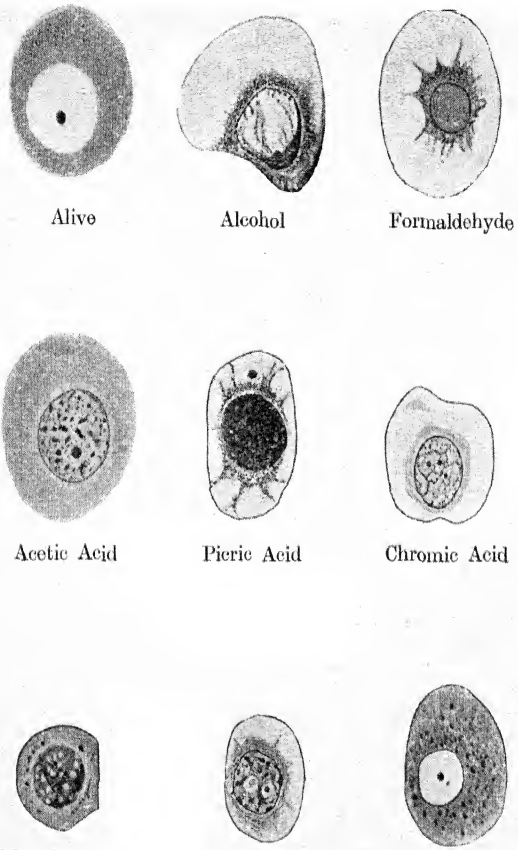


FIG. 2.—The Spermatogonium of the Salamander, as it exists during life and as it appears in section after various fixatives

* The figure labelled Acetic Acid represents a cell still lying in the fixative, not a section

(*From papers by* Tellyesniczky (1898 *and* 1905), *by kind permission of Messrs. Julius Springer*)

brane. The outer part of the cell is left empty. The contents of the nucleus are affected in much the same way. Towards the centre of the piece of tissue this effect is less marked, because the alcohol has been diluted by the water contained in the tissue.

Strangeways and Canti worked with cells with very delicate processes. As was explained in the last chapter, they described the cells while they still lay in the fixative, not after sectioning. It is natural that fine spongeworks, of the sort described by Hardy, cannot be seen except in very thin sections. Absolute alcohol caused a granular appearance throughout the cytoplasm and considerable shrinkage of the cell processes, while fat globules ran together and then dissolved, leaving spaces in the protein precipitate. Mitochondria were dissolved away. More shrinkage was caused than by any other fixative except chromic acid.

Formaldehyde

Suitable concentration for fixation : 4%–10%. *Osmotic pressure at* 4% *:* 32 *atmospheres.* (*Mammalian blood :* 6·7 *atmospheres.*)

Formaldehyde, HCHO, is a gas. It is sold as a colourless solution in water, containing nearly 40% of formaldehyde. This solution is called formalin. The formaldehyde given off from it is extremely irritating to the eyes and nose.

Formaldehyde was introduced into cytology by F. Blum (1893), who had previously discovered its antiseptic properties. He discovered its hardening properties accidentally on his own fingers. One day he slit up a mouse that was infected with anthrax and left it overnight in a solution of formaldehyde (presumably as an antiseptic). In the morning he was surprised to find that it felt as hard as though it had been preserved in alcohol. He then started a systematic study of its effects on tissues, trying it at 4%

on various tissues, including liver, kidney, the mucous membrane of the stomach, and brain. He found that it hardened tissues faster than alcohol, and preserved their external form better, so far as could be seen with the naked eye. He then embedded liver, kidney and mucous membrane in celloidin, sectioned them, and found the cells well fixed and capable of being stained in haematoxylin and synthetic dyes.

Formaldehyde is prepared by passing the vapour of methyl alcohol with air over strongly heated copper gauze. The copper catalyses the oxidation of the alcohol. The products of the reaction are formaldehyde (i.e. methylaldehyde) and water. These, passing together into a receiver, constitute formalin. A solid white condensation product, paraformaldehyde, forms slowly in formalin.

The concentration of solutions of formaldehyde should always be stated as percentage of formaldehyde, and not as percentage of formalin. '10% formalin' may mean 10% formaldehyde, and it may mean 4% formaldehyde.

It is convenient to give a general discussion of the effect of osmotic pressure under the heading of formaldehyde, because authors have stressed the necessity of making up formaldehyde with isotonic saline. Do fixatives with high osmotic pressures shrink cells, and those with low osmotic pressures swell them? Mr. Crawford's and my osmotic pressure determinations are given under the headings of the various fixatives in this and the next chapter. 5% acetic acid *swells* tissues, yet it has a pressure of 20 atmospheres, about three times that of mammalian blood (and hence, presumably, of the fluid of most mammalian cells). Saturated picric acid has a pressure of only $2\frac{1}{2}$ atmospheres, roughly a third of that of mammalian blood: yet picric acid generally shrinks cells badly. What is the cause of the swelling in one case and the shrinkage in the other? Clearly it is not osmotic pressure, for that would work

in precisely the opposite direction. Now picric acid is a strong precipitant of the proteins of the cytoplasm, while acetic acid does not precipitate them at all. It is precipitant action, and not high osmotic pressure, that causes shrinkage. $\frac{1}{2}\%$ chromic acid, with the very small osmotic pressure of 1·4 atmospheres, shrinks the cytoplasm badly : Bouin's fluid (p. 65), with the enormous pressure of about 100 atmospheres, shrinks much less or not at all.

For some unexplained reason, many authors have thought it proper to dilute 40% formaldehyde to the concentration required with 0·9% sodium chloride solution rather than with water. In doing so they have completely neglected the osmotic pressure of the formaldehyde itself. If 40% formaldehyde is diluted to 4% with water, the osmotic pressure gradually rises from about 23 atmospheres the moment after dilution to a fairly stationary figure of about 32 atmospheres the next day. (The gradual rise is presumably due to the formaldehyde being polymerized when at 40%, and gradually becoming depolymerized after dilution. Fluids containing formaldehyde do not maintain an absolutely constant osmotic pressure.) We see, therefore, that if we dilute with *water*, the 4% solution is itself strongly hypertonic, having nearly five times the osmotic pressure of mammalian blood. If we dilute with 0·9 sodium chloride instead of water, we have a fluid with an osmotic pressure of 39 atmospheres. It is obvious that the sodium chloride makes the fluid even farther from being isotonic with the tissues.

It should be mentioned that Carleton (1922) found dilution with isotonic saline slightly preferable ; but it appears to me that the subject requires reinvestigation. It might be advantageous, for some unexplained reason, to have formaldehyde solutions made very highly hypertonic ; but if so, there can be no likelihood of a 0·9% saline solution having any particular merit, rather than any other arbitrarily

chosen concentration. The salt may be helpful in some quite different way, if it really is helpful.

Blum (1927) tried concentrations of formaldehyde from 0·4% to 40%, and preferred 4%. Sjöbring (1900) recommended 8–10% as isotonic, but in this he was manifestly wrong. Bang and Sjövall (1916) found the best preservation of mitochondria with 10% (or stronger) formaldehyde, diluted from 40% with isotonic saline. This is of course a very hypertonic solution.

Formaldehyde is easily oxidized to formic acid, and therefore should not be mixed with chromic acid, potassium dichromate (see p. 45) nor osmium tetroxide. The oxidation proceeds slowly even when formalin is kept by itself in a closed bottle, and it is therefore a good plan to keep some powdered calcium carbonate at the bottom of the bottle, to neutralize the formic acid as it forms. This does not result, however, in absolute neutrality. The solution has a *p*H of about 6·4 (Romeis, 1928). This slight acidity is of no moment. For many purposes the acidity of unneutralized formalin is not objectionable.

The effect on proteins may be tested thus. Put 5 c.c. of albumin solution in a test tube. Add 5 c.c. of neutralized 8% formaldehyde. The concentration in the tube is now 4%. No precipitate is formed, even after standing for a day. Repeat the experiment with acidified formaldehyde (4·5 c.c. of 8% formaldehyde with 0·5 c.c. of pure acetic acid). The result is the same.

Repeat the experiment with neutral formaldehyde, using nucleoprotein solution instead of albumin. There is no precipitate. (A solution acidified with acetic acid gives a precipitate, but it is caused simply by the acid.)

Fischer found that even 40% formaldehyde has only medium powers of precipitating proteins, and 4% none at all. Berg obtained a slight precipitate of nuclein at 5%.

Are we to conclude that formaldehyde at ordinary concentrations is without effect upon proteins ?

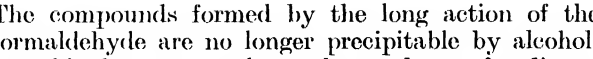
Put 1 c.c. of *undiluted* egg-white in a test tube. Slant the tube and carefully add 10 c.c. of neutralized 4% formaldehyde with a pipette, avoiding mixture of the egg-white with the formaldehyde solution. The egg-white lies at the bottom. Cork the tube and leave it for a week. The egg-white still lies at the bottom, undissolved but still soft. Formaldehyde does not harden the proteins of egg-white. Remove the supernatant fluid with a pipette. Add 10 c.c. of distilled water. Shake. The egg-white dissolves. (A small precipitate, presumably of globulin, falls on standing.) Has formaldehyde no effect upon proteins ?

Repeat the experiment, but this time carefully add 10 c.c. of absolute alcohol at the end, instead of 10 c.c. of water. As a control, put 1 c.c. of egg-white in another tube, and add 10 c.c. of absolute alcohol in the same way. Leave the two tubes for two days. The egg-white which has not been in the formaldehyde is now an opaque white, and firm. The egg-white which was previously in the formaldehyde is translucent and still soft. Pour away the alcohol and add 10 c.c. of distilled water. The egg-white does not dissolve. Leave it for a day. It swells somewhat, but does not dissolve. Shake. It dissolves. (A small precipitate falls on standing.)

These experiments show that formaldehyde does not harden albumin, nor render it insoluble in water. What it does do is to render it no longer capable of being hardened by alcohol. We shall see below that formaldehyde hardens tissues strongly. It appears that this must be due to an effect upon the lipides of the cell-membrane, since the substance of the cell is not hardened.

Formaldehyde exerts its effect upon proteins by a chemical change. If we take the simplest amino-acid of all, glycocoll, instead of a protein, to illustrate

what happens, the reaction is probably to be represented thus :



The compounds formed by the long action of the formaldehyde are no longer precipitable by alcohol, but this does not apply to the products of ordinary short fixation, for great shrinkage often takes place during dehydration after formaldehyde fixation.

It is generally allowed that formaldehyde does not destroy nor preserve fats, and exerts some fixative effect upon phospholipides. Millot and Giberton (1927), however, find that all lipides gradually disappear from tissues preserved in formaldehyde, the disappearance from fish liver being complete in three months. They think that a lipase present in the cells is not destroyed by this fixative. Kasarinoff (1910) states that formaldehyde alone does not preserve compound lipides, but the addition of 5% of Carlsbad salt achieves this end. (Carlsbad salt is chiefly sodium sulphate and bicarbonate.)

Formaldehyde is generally regarded as penetrating fairly rapidly, but taking rather a long time to exert its full effects. Underhill found that it penetrated more slowly than any other fixative (0·09 mm. in a quarter of an hour) with the possible exception of potassium dichromate. Tellyesniczky found the speed moderate ($2\frac{1}{2}$–5 mm. in twelve hours) as compared with his other results. It will be noticed that with formaldehyde Underhill's and Tellyesniczky's figures agree. Long fixation (one or two days) is indicated by the slow action on proteins. Tarkhan found that formaldehyde generally produces no shrinkage, but marked shrinkage occurs in 90% and absolute alcohol afterwards. (See above.) Patten and Philpott found an

average *increase* in length of 5% in pig embryos after fixation in '10% formalin' (whatever that may mean). By the time, however, that they were embedded in paraffin, they had shrunk to 79% of their original strength. Berg found a minute shrinkage (1%) in volume after fixation, but a shrinkage of 32% by the time the tissue was in paraffin.

Wetzel found 10% formaldehyde next after alcohol in hardening effect. After thorough washing, the resistance to bending was 1,700 (alcohol 4,500). After four days in 80% alcohol he found the tissue actually softer. Formaldehyde and osmium tetroxide are the only fixatives which do not result in the tissue subsequently becoming distinctly harder in alcohol.

No special washing out is required after formaldehyde fixation. Schmidt (1910) discovered that tissues that have been unduly hardened by formaldehyde may be softened by soaking in 10% citric acid. Tissues should be transferred direct from the fixative to 70% or 90% alcohol. The experiments described above show that water is best avoided. Staining in carmine and acid dyes is difficult. It has been shown (Stiasny, quoted by Jordan Lloyd, 1926) that proteins combine less easily with acids after combining with formaldehyde.

Fig. 2 shows the great shrinkage that may be caused by dehydration and embedding after fixation in 10% formaldehyde. Strangeways and Canti found that the fixative itself at 4% preserves the shape of the cell well, without shrinkage even of the delicate processes of tissue-culture cells. Fat was unchanged. Mitochondria, previously threads, became converted into rows of spheres. The outline of the nucleus became less clear than during life. Noël and Mangenot (1922) claim that formaldehyde gives very life-like fixation of the nucleus, unlike the standard nuclear fixatives. For some unexplained reason it is an atrocious fixative for the Mammalian testis. Shrinkage is so great that the sections are scarcely recognizable.

Acetic Acid

Suitable concentration for fixation : 0·5–5%. *Osmotic pressure of* 5% *solution :* 20 *atmospheres.* (*Mammalian blood :* 6·7 *atmospheres.*)

Acetic acid, CH_3COOH, is the essential constituent of vinegar, which is a 4% to 10% solution. The pure acid is a colourless liquid with a pungent smell. On cold nights it often freezes in the laboratory, and its resemblance to ice has given the name ' glacial ' to the pure acid, for solutions do not freeze readily. Solid acetic acid melts at about 17° C. Acetic acid can be mixed with water and alcohol in all proportions.

The use of acetic acid for the preservation or ' pickling ' of organic matter has been known since ancient times. I have not been able to find out who first used it in the study of cells. Remak (1854) was using it in 1854 for multinuclear cells in the rabbit's liver, and it is clear from his remarks that its action in showing up nuclei was already well known. Twenty years later Auerbach (1874) published a careful study of its action on the nuclei of the liver of the carp. Its position in cytological technique was established by Flemming, who included it in his famous fluid. (See p. 67.)

Acetic acid is made by the distillation of wood in the absence of air. A thick dark-brown watery fluid called pyroligneous acid distils over, as well as wood tar. Pyroligneous acid is a complex mixture containing acetic acid. It is redistilled, the hot gases passing through an emulsion of calcium oxide. The acetic acid is caught and dissolves as calcium acetate, while the other components of the mixture pass through. The solution of calcium acetate is evaporated and distilled with sulphuric acid. Acetic acid passes over. Further treatment frees it from water and other impurities. Vinegar is made by exposing weak wines to the air. A bacterium oxidizes the alcohol to acid.

Acetic acid may be mixed with any of the fixatives mentioned in this chapter.

The following tests show the reactions with proteins. Put 5 c.c. of albumin solution in a test tube and add 5 c.c. of 10% acetic acid. The acid is now at 5%, the most usual strength in fixing mixtures. No precipitate is formed. Next day there is still no sign of a precipitate. Repeat the experiment, using nucleoprotein solution. There is an immediate precipitate, consisting at first of an opalescence with fine particles. Two days later the particles have mostly fallen to the bottom. Remove the supernatant fluid and add 10 c.c. of distilled water. The precipitate does not dissolve. Add 1 c.c. of 10% potassium hydroxide and shake. The precipitate dissolves completely.

Fischer got precipitates with both albumin and globulin, but these were soluble in excess of acetic. In practice the fixative is always present in excess. Fischer and Berg both got nuclein precipitates, but differed as to the solubility of the precipitate in water.

We have seen that acetic acid does not fix the proteins of the cytoplasm. Why, then, is vinegar so extensively used for pickling? The reason is that the acidity in itself prevents decay. Few bacteria grow below *p*H 5, autolysis is reduced below *p*H 4·5, and moulds are checked about *p*H 3. This is preservation but not fixation.

Acetic acid has no effect on fats, which will dissolve out subsequently during dehydration unless steps are taken to preserve them. Its action on other lipides is uncertain. Mitochondria will be considered below.

Underhill found that acetic acid penetrated enormously faster than any other fixative, at a rate corresponding to 12 mm. in a quarter of an hour. Tellyesniczky found nothing approaching this figure, but it was among the fastest of those which he studied (4–5½ mm. in twelve hours). As was mentioned before, the rapidity of penetration must be attributed

to its not fixing the cytoplasm, and therefore not forming a barrier to its own diffusion. Short fixation (an hour or less for small pieces) suffices.

Pickled foods swell unless steps are taken to prevent it, and this is an effect of acetic acid which makes itself very evident in cytological technique, where one of its chief uses is in preventing the shrinkage caused by other fixatives. This effect, which is common to many acids, appears to be due to the formation of a salt between the protein and the anion (Jordan Lloyd, 1926, and Bayliss, 1923). The salt dissociates into a non-diffusible protein cation and an acetate ion. The latter cannot diffuse away, because it is held to the non-diffusible protein ion by electrostatic attraction. It exerts an osmotic pressure and thus sucks water into the cell and causes it to swell. As we shall see, acetic acid penetrates more rapidly than other fixatives. Therefore, when it is used in mixtures, the other components shrink a cell which has already been swollen by the acetic. An approach to the normal size is obtained. White connective tissue fibres, however, are permanently distorted by the swelling.

Since the cytoplasm is not fixed by acetic acid, it is not surprising that it gives less resistance to bending than any other fixative. After forty-two hours' fixation Wetzel's figure was only 9 (alcohol 4,500). After four days' subsequent hardening in 80% alcohol, the figure had only risen to about 50. It is clear that acetic acid not only does not harden tissues itself, but also prevents their subsequent hardening by alcohol.

No special washing out is required after acetic acid. Tissues may be transferred to 50% or 70% alcohol. They are not made particularly easy to stain by acetic acid, nor are they rendered unstainable.

Fig. 2 shows a cell in acetic acid. This figure differs from all the other figures except that of the living cell, because the others are of sectioned and stained cells. It will be noticed that the cytoplasm is scarcely

affected, but the chromatin is precipitated in a most unlife-like way. Acetic acid is an excellent fixative for showing up nuclei clearly for histological work, but for a cytological study of the interkinetic nucleus it is useless. Chromosomes, however, it preserves excellently, and acetic acid is a component of all fixatives for chromosome studies. They appear more sharply defined and life-like after acetic acid than after any other fixative. Nevertheless it is not proper to use acetic acid (except perhaps very dilute) in studying the condensation of the chromatin to form chromosomes, nor the telophase transformation of the chromosomes into the interkinetic nucleus.

Much has been written upon the destructive effect of acetic acid on mitochondria. Gatenby (1917 A and B) has particularly stressed that it should be omitted completely from mitochondrial fixatives. (See next chapter under Flemming's fluid.) Although in many cases it does destroy them, yet in others they have been shown to be resistant to it. The mitochondria of the mammalian spermatid are particularly resistant. Young (1928), working with the liver and kidney of Vertebrates, has shown that 5% acetic acid does not destroy mitochondria in the presence of potassium dichromate, except in birds. Nicholson (1916) found that the addition of 1% of acetic to a mixture of formaldehyde and potassium dichromate destroyed mitochondria in some brain cells, but not in others. Ciaccio (1909) includes acetic acid at 5% in his fixative for the microchemical demonstration of conjugated lipides, of which mitochondria are supposed largely to consist. Champy (1911) and Romeis (1913) have both seen mitochondria after acetic acid fixation, though Champy strongly advises its omission. I (1932) have demonstrated mitochondria in the liver of the newt and the mammalian kidney even after Carnoy's fluid. (See next chapter.) If one is working at a cell which has not been investigated, it is best to omit acetic acid at first. Later one may find the

mitochondria unaffected by it. If so, its addition will prevent shrinkage. In work on spermatogenesis, it will make it possible to define the exact stage of the cell by precise fixation of the chromosomes. Acetic acid, then, may or may not destroy the mitochondria ; but there is no evidence or likelihood that it ever preserves them, for it neither hardens lipides nor precipitates proteins other than nucleoproteins and mucin.

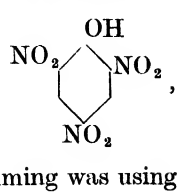
Strangeways and Canti found that 5% acetic acid made the thread-like mitochondria of their tissue-culture cells become faint rows of granules. Lewis and Lewis (1915), using the vapour of weak acetic acid on tissue cultures from chick embryos in hanging drops, similarly found that the threads changed into rows of swollen vesicles.

The action of acetic acid on Golgi bodies has not been properly studied, but it is omitted from fixatives for them. At ordinary concentrations it probably destroys them.

Zirkle (1928 B) has shown that the fixation images produced by ferric, chromic, mercuric and copper acetates are the same as those of acetic acid, despite the differences of *p*H. Working with root tips of maize, he found chromosomes well fixed and mitochondria not preserved.

Picric Acid

Suitable concentration for fixation : saturated solution. Osmotic pressure of saturated solution : 2·5 atmospheres. (*Mammalian blood : 6·7 atmospheres.*)

Picric acid,  , is a yellow crystalline solid. Flemming was using picric acid in 1882, but I

do not know who introduced it into cytological technique. It is a sufficiently strong acid to decompose carbonates. Its solubility in water varies from about 0·9% to 1·2% according to the temperature of the room. It is soluble in alcohol, benzene and xylene. It is used as an explosive under the name of Lyddite. It burns quietly when not confined, but explodes violently when detonated. To prevent accidents it is usually kept just moist with water. In this condition it is perfectly safe to handle. It is said to be possible to eat a gram without permanent damage (Romeis, 1927). It is prepared by dissolving phenol in sulphuric acid, adding nitric acid, and heating. It is convenient to keep it as a saturated solution, with excess at the bottom of the bottle. The exact concentration will vary with the temperature, but not sufficiently to interfere with the action of the mixtures made up with it. It may be mixed with any of the fixatives mentioned in this chapter.

The following experiments show its effects on proteins. Put 1 c.c. of albumin solution in a test tube and add 9 c.c. of a saturated aqueous solution of picric acid. A precipitate is formed instantaneously. When the precipitate has fallen to the bottom, remove the supernatant fluid and add 10 c.c. of distilled water. The precipitate does not dissolve. Throw the contents of the tube into a measuring cylinder containing 490 c.c. of distilled water. The yellow protein precipitate will be found at the bottom next morning. Albumin precipitated by picric acid is scarcely, if at all, soluble in water, the usual statements to the contrary notwithstanding. Repeat the experiment, using nucleoprotein solution. There is an immediate coarse precipitate, which falls completely within a quarter of an hour. Remove the supernatant fluid and add 10 c.c. of distilled water. The precipitate does not dissolve.

Fischer got precipitates with albumin, globulin, nuclein and nucleic acid. The latter was soluble in

water, the others insoluble. Berg found picric acid only a moderate to weak precipitant of nuclein. It is said (Jones, 1920) that picric acid precipitates the protein only from nuclein solutions, leaving the nucleic acid in solution. In general, we may conclude that tissues fixed in picric acid may be brought into water without danger.

The action of picric acid on proteins is different from that of the fixatives we have so far discussed. It is well known that complex anions precipitate proteins, and indeed they are used as tests for the presence of proteins. Phosphotungstic acid is one of these, and picric acid is another. It appears that the anion forms an actual chemical compound, a protein picrate.

Picric acid appears to be without effect, harmful or preservative, on lipides.

Underhill found that 0·7% picric acid penetrates at medium speed (0·24 mm. in a quarter of an hour). Tellyesniczky found it slow (1–2 mm. in twelve hours) in comparison with the other fixatives he tried. A few hours' fixation generally suffices for small pieces. Berg found that picric acid caused greater shrinkage of liver than any other fixative, namely a reduction of 26% in volume, followed by further reduction during dehydration and embedding, leaving it finally with a volume only 42% of the original. Tarkhan also reports shrinkage. Kaiserling and Germer report shrinkage with great distortion. Wetzel found that saturated picric acid has much less hardening effect than any other vigorous protein precipitant. After thirty-six hours' fixation the figure for resistance to bending was only about 70 (alcohol 4,500). After four days' subsequent immersion in 80% alcohol, it was only about doubled.

Picric acid may be washed out in 50%, 70%, or 90% alcohol. It is usually said that it must on no account be washed out in water, but this statement rests on an erroneous idea as to the solubility of albumin picrate.

Generally no special washing out is necessary, for the yellow excess of picric acid is taken out during ordinary dehydration, and the yellow colour left in the tissues is not harmful in itself, nor does it prevent subsequent staining. Staining is easy after picric acid fixation. The amount of picric acid left in the tissue is small, because it is extracted by most of the solvents used in ordinary technique (water, alcohol, benzene, xylene). Should it be desired to remove all traces of yellowness a few drops of a saturated aqueous solution of lithium carbonate may be added to the dehydrating alcohol (70% or 90%). A cloudiness is produced, which disappears as the very soluble lithium picrate is formed. More drops of lithium carbonate solution are added until the cloudiness no longer disappears. The removal of the excess of picric acid is then complete. The extraction may be done, if preferred, when the tissue is in sections on the slide. The lithium carbonate technique was introduced by Jelinek (1894). Romeis (1927) recommends the use of calcium chloride solutions for the removal of picric stains from the fingers. I find that rather prolonged soaking is necessary.

Fig. 2 shows a cell fixed with saturated picric acid. The extreme shrinkage is evident. Strangeways and Canti found that small fat globules fuse to form large masses. This is probably to be explained by the distortion of the cytoplasm. Thread-like mitochondria become faint rows of spheres.

Chromic Acid

Suitable concentration for fixation : $\frac{1}{2}$%. *Osmotic pressure of* $\frac{1}{2}$% *solution :* 1·4 *atmospheres.* (*Mammalian blood :* 6·7 *atmospheres.*)

Chromium trioxide, CrO_3, is the anhydride of chromic acid, H_2CrO_4, which is formed when the oxide is put into water, but which cannot be isolated. The

oxide forms light-red or brownish crystals, and its solution is brownish yellow.

Chromic acid was introduced into cytology by Hannover in 1840. His paper is in the form of a letter to Professor Jacobson, who had introduced chromium into therapy. Hannover had been staying with Jacobson in Copenhagen. On the day of his departure, Jacobson had shown him a divided mammalian eye, preserved in chromic acid. Hannover was struck by the good preservation. He had been looking for a fluid which would preserve both outward form and internal structure. In chromic acid he found a substance which not only achieved both, but also hardened the object so that fine sections could be made. He mentions a large number of tissues which were fixed in a life-like condition, including blood-corpuscles, cartilage cells, medullated nerve-fibres, and ciliated epithelium from the mouth of a frog. He generally mixed one part with 16 to 20 of water.

Chromium trioxide is extremely soluble in water. 100 grams of a saturated solution contain 62 grams of it. It is readily deliquescent, so it is best to keep it in the form of a solution of known strength, preferably 1%. It is a weak acid which is displaced from its salts by acetic.

Chromium trioxide is prepared by treating a dichromate with sulphuric acid. The manufacture of potassium dichromate is discussed in the next section.

Chromic acid is an oxidizing agent, being readily reduced to Cr_2O_3. It must not be mixed with reducers, such as alcohol and formalin. It is a powerful precipitant of proteins, as these experiments will show.

Put 5 c.c. of albumin solution in a test tube, and add 5 c.c. of 1% chromic acid. A precipitate forms instantaneously. Next day the whole of the contents of the tube are in the form of a sponge, with the fluid held in its meshes. Pour away as much of the fluid as possible, and throw the precipitate in 500 c.c. of

distilled water. It does not dissolve. Repeat the experiment, using nucleoprotein solution. There is an immediate coarse precipitate, nearly all of which falls to the bottom within fifteen minutes. The precipitate is insoluble.

Fischer got insoluble precipitates with albumin, globulin, nuclein and nucleic acid. Berg found it a moderate or weak precipitant of nuclein.

The fixative powers of chromic acid are used in industry. In ordinary tanning the precipitant used is tannin, but for certain purposes, especially for the uppers of boots and shoes, chrome-tanned leather is preferred. It is more resistant to moisture and high temperature than ordinary leather. In tanning the chromic acid is fixing not albumin nor globulin nor nuclein, but the collagen of white connective tissue fibres. Collagen is an insoluble protein, but fixation is necessary nevertheless, for the fibres swell up and putrefy if a skin is kept wet, while if it is dried they adhere together and elasticity is thus lost. Potassium dichromate and sulphuric acid are used together. The tanning is produced by the chromic acid resulting from their interaction.

Chromic acid partly dissociates in water into H and $HCrO_4$. Berg (1927 A) thinks that it fixes in two stages. He distinguishes a primary action, in which the protein is denatured and precipitated, and a secondary action, in which it is hardened. According to Berg, the anion $HCrO_4$ is concerned in the secondary action.

It appears that in chromic acid fixation there is chemical combination with the proteins. Exactly what happens is not known, but part of the process is an oxidation, resulting in the formation of Cr_2O_3, which gives a greenish colour to the tissue. This is certainly not all that happens, for oxidizing agents are by no means necessarily fixatives.

Fats are unaffected by chromic acid. There is no fixative action on lipides.

Underhill found that $\frac{1}{2}\%$ chromic acid penetrates at a medium speed (0·22 mm. in fifteen minutes). Tellyesniczky found that 1% chromic acid penetrates generally 2–3 mm. in twelve hours, but more slowly into brain. Twelve hours or a day's fixation is generally indicated. Berg found shrinkage of 22% in the volume of liver fixed in 1% chromic acid, followed by a further 14% during dehydration and embedding. This is moderate shrinkage. Tarkhan found only slight shrinkage at the same concentration. Wetzel found that it hardens moderately. His figure for resistance to bending after thirty-six hours' fixation and thorough washing is about 230 (alcohol 4,500). After the standard four days in 80% alcohol it was increased to rather over 400.

The washing out of chromic acid is important, because precipitates will appear if it is not done carefully, and the tissue will be difficult to stain. Twelve hours in running water suffice for pieces of the size ordinarily used in cytology. If tissues are transferred directly to alcohol from chromic acid, a precipitate of the insoluble suboxide is formed, though this is said to be avoided if the alcohol is kept in the dark.

Chromic acid leaves the tissues in a state in which they may readily be stained by basic dyes.

Fig. 2 shows the great shrinkage of the cytoplasm caused by $\frac{1}{2}\%$ chromic acid. It is reduced to a vestige surrounding the nucleus. The chromatin is precipitated in the form of a coarse net. Strangeways and Canti found with $\frac{1}{2}\%$ chromic acid a heavy coarse precipitate in both nucleus and cytoplasm, with shrinkage of cell processes. Mitochondria were not visible, and fat globules fused together. As with picric acid, it seems probable that the fusion of the fat globules is caused by the destructive precipitation of the cytoplasm, and not by any action on the fat. Possibly many fixatives destroy mitochondria in the same way. Strangeways and Canti found that cells

fixed in chromic acid dissolve when the light of the dark-ground condenser is allowed to play on them. The solution is finally complete, nothing remaining except the fat globules. Similarly, Berg (1927) notices that the outsides of pieces of tissue sometimes dissolve away after chromic fixation, owing to the action of light.

Potassium Dichromate

Suitable concentration for fixation: 1·5%. *Osmotic pressure of* 1·5% *solution:* 3·3 *atmospheres.* (*Mammalian blood:* 6·7 *atmospheres.*)

Potassium dichromate, $K_2Cr_2O_7$, forms large red poisonous crystals. It was introduced into cytological technique by Heinrich Müller at a meeting of the Würzburg Medical Society in 1859. He had used it, mixed with sodium sulphate, in a study of the smooth muscle and nerve-plexuses in the choroid of the human eye (1872). 'Muller's fluid' is 2–2½% dichromate, with the addition of sodium sulphate. To this day his mixture is often used, though the sulphate is without effect.

Chromium occurs in nature as chrome iron ore. Chromium is a hard metal which is not readily oxidized. It is used in steels to impart hardness, and also as a plating substance over nickel. The ore gives potassium chromate when roasted with potassium carbonate. The chromate is transformed to the dichromate by treatment with the appropriate amount of sulphuric acid. Too much acid would convert part of it into chromium trioxide. (See last section.) The dichromate is usually called bichromate, but this is clearly erroneous, for such a name could only apply to potassium hydrogen chromate, which does not exist.

Potassium dichromate is soluble to about 9% in water. Aqueous solutions are slightly acid, because it dissociates partly into $2K + Cr_2O_7$, and the latter

gives a small amount of $H + CrO_4$ (Berg, 1927 A). The dichromate is a strong oxidizing agent, though not so strong as chromic acid. It should not be mixed with alcohol nor formaldehyde. Regaud's fluid, a fixative for mitochondria, consists of a mixture with formaldehyde, but it presents no advantages over formaldehyde fixation followed by treatment with dichromate (Zirkle, 1928 A). It is convenient to keep dichromate in solution at 3% or 5%. Its action on proteins may be studied as follows.

Put 5 c.c. of albumin solution in a test tube and add 5 c.c. of 3% potassium dichromate. No precipitate is formed, nor does any appear later, though the tube be kept for days. Repeat the experiment, using nucleoprotein solution. The result is the same. Potassium dichromate by itself is not a precipitant of proteins.

Repeat both experiments, using acidified dichromate (4·5 c.c. of 3% dichromate with 0·5 c.c. of pure acetic acid). The difference in the results is startling. Neither dichromate nor acetic acid precipitates albumin, but together they form a dense precipitate instantaneously. The precipitates resemble those formed by chromic acid.

Fischer obtained no precipitates with albumin, globulin, nuclein and nucleic acid, when unacidified dichromate was used. He found no precipitate with nuclein and nucleic acid, even in acid solution. This disagrees with my results with nucleoprotein.

Unacidified potassium dichromate is not a precipitant of proteins. How, then, does it fix? Put 1 c.c. of *undiluted* egg-white in a test tube. Slant the tube, and very carefully introduce 10 c.c. of 1·5% potassium dichromate solution with a pipette, taking care to avoid mixing the egg-white with the dichromate as far as possible. The egg-white should lie below the fixative solution. Cork the tube and set it aside for ten to twelve days. At the end of this period the egg-white is still soft, but undissolved. It lies at the bottom of the tube. Pour away as much

of the dichromate as possible and gently fill the tube with distilled water. Pour the water away and repeat this washing process twice. Then fill the tube with distilled water and leave it. Days later the egg-white will still be undissolved. Potassium dichromate renders albumin insoluble, but does not precipitate it. It resembles osmium tetroxide in this respect. They exert their effects by a slow action which changes the proteins so that they are no longer soluble in water, nor precipitable as coarse spongeworks by alcohol. The life-like homogeneity of the cytoplasm is preserved. The dichromate has the disadvantage of dissolving chromatin, and it must be omitted from fixatives for chromosomes (unless acidified).

Dichromate fixes in two entirely different ways, according to whether it is acidified or not. The whole matter has been carefully worked out by Zirkle (1928 A). He finds that all dichromates fix like chromic acid when they are more acid than about *p*H 4·6, and all fix in an entirely different way when less acid or more alkaline than that. (Actually the critical *p*H varies between *p*H 4·2 and 5·2.) When more acid than the critical figure, chromosomes are well preserved, cytoplasm and chromatin are both precipitated as nets, and mitochondria are not preserved. When it is less acid than the critical figure, chromosomes are dissolved, no chromatin network appears, the cytoplasm is excellently and homogeneously preserved, and the mitochondria are well fixed. The differences between different dichromates depend simply on the *p*H of their solutions. Calcium and copper dichromates fix like chromic acid: the dichromates of potassium, ammonium and zinc fix in the other way, because their solutions are less acid. The differences between the different dichromates had been known for a long time (Burchardt, 1897), before the days of *p*H, but it remained for Zirkle to explain it. Tellyesniczky (1898) long ago sought to fix every part of the cell perfectly by mixing

acetic acid (for the nucleus) with potassium dichromate (for the cytoplasm). It is obvious now why he failed. Unacidified potassium dichromate is a splendid fixative for the cytoplasm and mitochondria, but when acidified it has no advantages of any sort over chromic acid.

To speak of 'chrome fixation' is clearly absurd, for it includes two mutually exclusive processes. No two fixatives are more unlike than chromic acid and unacidified potassium dichromate. It is now clear also that chromic acid and potassium dichromate should not be mixed. For this reason Altmann's fluid is to be preferred to Champy's. (See p. 70.)

Dichromate fixation of a protein is made use of in photography, when a thin film of gelatine is made insoluble in water.

The chemistry of fixation by dichromate is not understood. Mann (1902) appears to suppose that it decomposes into potassium chromate and chromium trioxide, and that the latter combines chemically with the proteins. Certainly the decomposition occurs, for the orange dichromate is converted into the yellow chromate, and for this reason plenty of fluid should be used, or the fluid changed. The chromate has no fixative powers.

Dichromate has no effect upon fats, but Ciaccio (1909) has shown that it preserves conjugated lipides, if allowed to act for a long time. This happens in the presence of 5% acetic acid. Mitochondria should therefore be preserved by dichromate even when acidified, but they might be much distorted by the precipitant effect of the acidified solution on the surrounding cytoplasm. Tellyesniczky supposes lipides to be dispersed throughout the cell, and thinks that the fixative power of this non-precipitant of protein is due to its action on these dispersed lipides (1927). The action on lipides is said to be an oxidation, but chromic acid, a stronger oxidizer, does not have the property of preserving them.

Tellyesniczky finds that dichromate penetrates moderately fast (2½–5 mm. in twelve hours). Underhill finds that it scarcely penetrates in fifteen minutes, but later it penetrates so rapidly that in an hour it has reached more than twice as far as most of the other fixatives reach in fifteen minutes. This result requires confirmation. Fixation should be long (days) on account of the slow action on proteins. Berg found no change in volume in fixing liver with 3% dichromate, but very great subsequent shrinkage, ending with a total shrinkage of 51% by the time it was impregnated with paraffin. Tarkhan found slight shrinkage with 4%, and swelling with 1%.

Potassium dichromate should be washed out with running water. The same remarks apply as to the washing out of chromic acid. Dichromate makes mitochondria particularly easily stained by acid fuchsin. Because it both preserves lipides and helps their subsequent staining, it is often useful to 'post-chrome' tissues for three days in a 3% dichromate solution, after fixation.

Fig. 2 shows the good preservation of the cytoplasm achieved by dichromate fixation. Vacuoles are seen in the nucleus, caused by the solution of the chromatin. Strangeways and Canti found with a 2% solution a tendency for fat globules to run together, and for the mitochondria to become swollen in breadth.

Mercuric Chloride

Suitable concentration for fixation: saturated solution. Osmotic pressure of saturated solution: 5·0 *atmospheres.* (*Mammalian blood:* 6·7 *atmospheres.*)

Mercuric chloride, $HgCl_2$, is sold as a white powder. The first mention I can find of its use in cytological technique is by Remak in 1854. It had previously been used for gross preservation. Remak only mentions using it at the extreme dilution of 0·2%.

He was studying multinucleated cells in the liver of the embryo rabbit. He mentions that it shrinks cells and nuclei slightly. He does not claim to have introduced the substance into cytology.

Mercury occurs naturally as the sulphide, cinnabar. The pigment vermilion is the pure sulphide. The chloride is prepared by heating a mixture of the sulphate with common salt. Mercuric chloride sublimes. Since it has a corrosive effect on mucous membranes, it used to be called corrosive sublimate. This old-fashioned name is still often used in zoological laboratories, but among chemists it has gone the way of dephlogisticated marine acid gas. Mercuric chloride is one of the most violent poisons known. Egg-white is a good antidote, for reasons which will become apparent shortly. Mercurous chloride is the purgative drug calomel.

When dissolved in water, mercuric chloride partly hydrolyzes into hydrogen and chlorine ions and $(HgCl)_2O$ or HgClOH. (See Luther (1904). The statements of Mann (1902) and Lee (see Gatenby and Cowdry, 1928) are incorrect.) The hydrogen ions make the solution slightly acid. It dissolves in water to about 7%, and is about five times as soluble in alcohol. It is more soluble in sodium chloride solutions than in water. It will dissolve at about 10% in 0·75% sodium chloride. Such solutions contain two distinct double salts. Convenient stock solutions are saturated solutions in water and in 0·75% sodium chloride. Mercuric chloride may be mixed with any of the fixatives mentioned in this chapter.

The powerful precipitant effect of mercuric chloride solutions on proteins is shown by these tests. Put 1 c.c. of albumin solution in a test tube and add 9 c.c. of a saturated solution of mercuric chloride in water. A precipitate is formed instantaneously. When it has sunk to the bottom, remove the supernatant fluid and add 10 c.c. of distilled water. The

precipitate is insoluble, and begins to fall to the bottom again in a few minutes. Add 2 c.c. of a saturated solution of sodium chloride or potassium iodide. The whole of the precipitate dissolves at once. (With the iodide there is a momentary appearance of the red mercuric iodide before the precipitate dissolves.) Repeat the whole experiment, using nucleoprotein solution instead of albumin. The result is the same all through, except that the precipitate does not form quite instantaneously.

The solubility of the precipitates in potassium iodide is of great importance in connexion with washing out. (See below.) Their solubility in sodium chloride makes one question whether it is logical to use mercuric chloride in solution with sodium chloride for fixation. The point may be settled practically. Put 1 c.c. of albumin solution in a test tube and add 9 c.c. of a saturated solution of mercuric chloride in 0·75% sodium chloride. A precipitate forms instantaneously. It is therefore logical to use such a solution as a precipitant fixative.

Acidification exerts a profound effect upon mercuric fixation, as the following experiments show. Put 1 c.c. of albumin solution in a test tube and add a mixture of 8·5 c.c. of saturated aqueous mercuric chloride and 0·5 c.c. of pure acetic acid. No precipitate is formed at once, but the fluid gradually becomes cloudy. The statement is commonly made (e.g. Spuler, 1927) that acetic acid increases the precipitating power of mercuric chloride, but it is untrue. Next day there is a considerable amount of fine white precipitate, which has not fallen to the bottom. Add 2 c.c. of saturated sodium chloride or potassium iodide. The precipitate does *not* dissolve. (Under *exactly* the same circumstances, with the mercuric chloride still present in the tube, the precipitate dissolves at once in the absence of acid.) Acetic acid makes fixation by mercuric chloride much less energetic, but gives insolubility in sodium chloride and potas-

sium iodide. Acidified mercuric chloride gives an immediate precipitate with nucleoprotein, as one would expect; but it resembles neither an acetic nor a mercuric precipitate, for it does not dissolve on the addition of potassium hydroxide or sodium chloride, in the amounts used in the previous experiments.

Fischer obtained precipitates with albumin, globulin, nuclein and nucleic acid, those with the latter appearing only slowly. All were insoluble in distilled water.

Before considering the special effect of mercuric chloride on proteins, we may notice the effect of salts in general. In concentrated solutions most salts precipitate proteins. The process is called 'salting out'. It appears that the salts compete with the proteins for the water molecules which are associated with the aggregates of protein molecules. If they extract the water, the protein is precipitated. Different anions have different powers of precipitating, irrespective of the cation. With egg-albumin citrates are the most effective precipitants, then tartrates, sulphates, acetates, chlorides, nitrates, and finally chlorates. (It is worth mentioning that in this 'Hofmeister series' the hydrogen ion concentration of the solution is not taken into account. Perhaps it is not only the anion that is important.) Could the sodium salts of these anions be used as fixatives? They could not, because the process is reversible. The precipitated protein is not changed permanently, and if placed in water or weak salt solutions it dissolves.

Certain cations, however, cause precipitation of proteins in an insoluble form, irrespective of the anion. These are the heavy metal cations, and mercury is an example. It was always thought till fairly recently that the salts of the heavy metals fixed by forming insoluble compounds with the proteins. Thomas and Norris (1925) have investigated the matter, using ferric chloride. There is no reason

to suppose mercuric chloride different. Extremely weak solutions do not precipitate albumin at all. If the concentration is increased, a slight precipitate appears. This is regarded as a compound, a real mercury albuminate. The albumin is acting as anion, because the still very weak solution of ferric chloride is on the alkaline side of the isoelectric point. If the concentration of the ferric chloride is increased, the precipitate dissolves, because the albumin becomes itself a cation when the *p*H of the fluid goes to the acid side of the isoelectric point. If the concentration of the ferric chloride is increased still further, an entirely new phenomenon appears. The albumin is precipitated in a denatured form, *not* as a compound with mercury. It carries down with it a good deal of mercuric chloride, but the amount of mercury varies and is in no special proportion to the amount of albumin, and it can be completely washed out with water. In fixation the mercuric chloride is always present in great excess, and therefore we are concerned only with this denaturation, and not with the compound formed by very dilute solutions. The denatured protein is insoluble. Thomas and Norris regard it as the same as the precipitate produced by heating, as when an egg is boiled. In the absence of heavy metal ions coagulation only takes place at high temperatures, but in the presence of high concentrations of heavy metal salts it takes place at the temperature of the room. It should be mentioned that albumin which has been dialyzed absolutely free from all salts cannot be coagulated by heating. All salts, then, assist denaturation, quite apart from the salting-out effect ; but only the heavy metal salts coagulate at room temperature. As we have seen, albumin precipitated by alcohol is also said to be denatured, but the precipitate differs from the mercuric precipitate by being insoluble in sodium chloride solution.

Mercuric chloride is without effect on lipides.

Underhill found 5% mercuric chloride the fastest penetrant of all she tried except acetic acid. Her mean figure is 1·7 mm. in fifteen minutes. Tellyesniczky found a much lower speed with a 7·5% solution ($2\frac{1}{2}$–4 mm. in twelve hours). He found that it penetrates 10–12 mm. into egg-white in three days. Short fixation (one to a few hours) is best with mercuric chloride. Precipitates form if it is prolonged. Berg found a reduction in volume of only 9% after fixation with the saturated solution, and a total reduction of 30% after impregnation with melted paraffin. 30% represents less final shrinkage than with any other fixative, but, as we shall see, the cell contents are often much shrunk. Tarkhan found considerable shrinkage. Wetzel found the hardening effect to be rather great. After thirty-six hours' fixation with a saturated solution, followed by thorough washing, the resistance to bending was rather more than 1,000 (alcohol 4,500), and four days' subsequent treatment with absolute alcohol gave an increase to more than half as much again.

Tissues fixed with mercuric chloride contain a precipitate which ruins the preparation unless it is removed. Its composition is not known. It is clearly not mercuric chloride, for this is quite soluble in water and very soluble in alcohol. It is probably either mercurous chloride or else a phosphate formed by reaction with the phosphates contained in the cells. It can be removed by iodine. The reaction is not understood. Since iodine is almost insoluble in water, but quite soluble in potassium iodide solutions, it would seem convenient to use a solution of iodine in potassium iodide for its extraction ; but the experiments described above show that the iodide would completely undo the fixation by rendering the proteins soluble once more (unless acetic acid were used in the fixative). Therefore a solution of iodine in 70% alcohol is convenient. It is best to keep a saturated solution in 70% alcohol in stock, and to

add a little of this to some 70% alcohol when it is required to wash a piece of tissue fixed in mercuric chloride. The exact concentration of the iodine is not a matter of importance. It is usually said that one should add enough of the saturated solution to make a fluid resembling port. I find that at no concentration does an iodine solution resemble port, but a solution resembling sherry gives good results. Strong iodine solutions must be avoided, as they make the tissue unduly soft. As the iodine does its work, it is gradually absorbed and the colour disappears. The fluid must then be replaced by a fresh solution, and the process repeated if necessary until the colour remains.

One great advantage of mercuric fixation is that it leaves the tissues in a state in which they may very readily be stained by any stain. Carmine is better after mercuric chloride than after any other fixative.

Fig. 2 shows the great shrinkage of the cytoplasm caused by mercuric fixation. The nucleus is fixed in a most unlife-like way. Strangeways and Canti found a coarse precipitate in both nucleus and cytoplasm. Small fat globules run together to form large masses of irregular shape. Mitochondria are not destroyed.

Cobalt Nitrate

Cobalt nitrate, $Co(NO_3)_26H_2O$, does not merit a long section to itself, firstly, because it is not used as a fixative in a general sense, and secondly, because its action is not in the least understood. Cajal discovered in 1912 that the Golgi element was subsequently more readily impregnated with silver if certain nitrates had been added to the formaldehyde fixative. He found uranium, lead and manganese nitrates all suitable, particularly the first. Da Fano (1920 A and C) found cobalt preferable.

Osmium Tetroxide

Suitable concentration for fixing : 1%. *Osmotic pressure of* 1% *solution :* 1·2 *atmosphere.* (*Mammalian blood :* 6·7 *atmospheres.*)

Osmium tetroxide, OsO_4, forms very pale yellow crystals. It is said to have been used as far back as 1849, but it was really introduced into cytological technique by Max Schultze in 1866. Schultze had already used it in a special way in a study of the phosphorescent organ of glow-worm. He first tried it as a fixative on the phosphorescent marine protozoon Noctiluca. He noticed that the animals died very quickly and could be preserved a long time in a life-like condition. He remarks on the darkening of the animal, caused by the reduction of the osmium. He used various concentrations, but gives no exact figures. The allied ruthenium tetroxide has recently been tried, and certain advantages are claimed for it (Carpenter and Nebel, 1931).

Osmium is related to platinum and occurs with this and other rare metals (including ruthenium) in platinum ore, all being in the metallic state. The ore is found in small grains in the Ural Mountains and elsewhere. Platinum forms about three-quarters of the ore. Osmium, like platinum, is a shining white metal, which is not oxidized by air at ordinary temperatures. It is the heaviest substance known. At very high temperatures it is oxidized to the tetroxide. The latter may also be made by the action of aqua regia on the metal, which is untouched by any acid acting alone. The tetroxide, at 17*s.* a gram, is one of the most expensive substances in the world. It is sold in small sealed glass tubes, each containing a definite weight of the substance. These are soaked in water till the labels come off, and then dried. Each is scored with a file and broken in two over a sheet of clean paper, which catches any crystals that may chance to fall. The crystals are dropped into a glass

stoppered bottle. Some of the crystals usually remain in the broken tube, so the tube is dropped into the bottle also. Water is added to make a stock solution at 2%. (It will dissolve up to about 6%.) The bottle is placed in a cardboard box, to prevent the action of light. Light causes rapid reduction to a black or brown lower oxide or hydroxide, except in the complete absence of dust. It is far better to keep it in an ordinary bottle in a box, than in a black bottle. In a black bottle one cannot see the contents. One does not know whether the solution is deteriorating for any reason, nor how much remains. This is very inconvenient, for it takes several days to dissolve. The result is that when one wants some osmium solution, one may have to wait several days for it. Heat must not be used to hasten solution, as it causes reduction.

Berg (1927 A) states that one drop of saturated mercuric chloride solution keeps 10 c.c. of 2% osmium solution unreduced for years, even in full daylight. I have tried this for months with complete success. Such solutions are very suitable for making up Mann's fluid (p. 71), but they must not be used for precipitation tests. The action of the mercuric chloride is not understood. The amount is insufficient for it to work simply as an oxidizer.

Osmium tetroxide and its solutions form a vapour with a faintly pleasant smell and a most damaging effect upon the eyes and mucous membranes. One must not hold open bottles near one's face. Brown or black stains are made on fingers by the solution unless one carefully avoids it. On account of its being volatile, osmium tetroxide solutions should be used in glass-stoppered vessels. Palmer (1930) has devised a colorimetric method of estimating the amount of it in solutions. He placed 2 c.c. of 2% osmium in each of three containers. One was a glass bottle with a ground-glass stopper. Another was a glass tube with a well-fitting cork. The third was a glass tube

covered with a glass plate. At the end of a week at about 25° C. the concentration of the tetroxide had fallen to 1·35% in the first, to 1·1% in the second, and to 0% in the third.

Osmium tetroxide is often called osmic acid, but this is quite absurd. It is not an acid : it is a non-electrolyte : it forms no salts : it is neutral to indicators. The only objection to its full title is its length, but it is perfectly legitimate to contract this by speaking of osmium solutions.

Osmium tetroxide is a strong oxidizer and must therefore not be mixed with alcohol nor formaldehyde.

The following experiments show its effects on proteins :

Put 1 c.c. of albumin solution in a test tube and add 1 c.c. of 2% osmium tetroxide. No precipitate is formed. The fluid goes brownish. Keep it in the dark. Next day there is still no precipitate. Repeat the process, using nucleoprotein solution instead of albumin. The result is the same. Unacidified osmium solutions do not precipitate proteins. Has unacidified osmium any effect at all ?

Put 1 c.c. of *undiluted* egg-white in a test tube. Slant the tube and very carefully add 10 c.c. of 1% osmium tetroxide solution with a pipette, avoiding mixture of the fluids. Cork the tube, vaseline the cork, and set aside in the dark for a week. Gently pour away the blackened fluid. A black jelly remains in the bottom of the tube. Slant the tube and half-fill it with distilled water with a pipette. Pour it away and half-fill once more with water. The previously fluid albumin has been transformed into a jelly which retains its shape when the tube is slanted. Gently test it with the point of a pipette. The jelly is very soft and readily broken. Cork the tube and shake hard. The jelly disintegrates into small lumps.

Repeat the experiment, but this time do not break up the jelly. Pour away the second lot of water and

half-fill with absolute alcohol. Cork and set aside for a few days. The alcohol replaces the water in the albumin jelly, and the latter becomes somewhat firmer, so that it gives an appreciable resistance to the entry of the point of a pipette. The jelly may be removed and cut into shapes with a knife. It is elastic and returns to its former shape when pressed and released.

Put 1 c.c. of albumin solution in a test tube and add 1 c.c. of 2% osmium tetroxide. Shake. Add 10 c.c. of absolute alcohol. No precipitate is formed. Evidently the osmium has instantaneously caused a profound change in the albumin, for alcohol is an energetic precipitant of albumin, as we have seen. This is one of the great virtues of osmium as a fixative: it does not make a homogeneous protein solution heterogeneous, and it prevents alcohol from having that effect subsequently. Cells fixed with osmium preserve the homogeneity of the living cell. The spongework appearance produced by protein precipitants is avoided.

It may be mentioned that osmium prevents coagulation not only by alcohol but also by heat (Berg, 1927).

Acidified osmium behaves in a rather different way. Put 1 c.c. of albumin solution in a test tube and add a mixture of 1 c.c. of 2% osmium tetroxide and three drops of pure acetic acid. There is no immediate precipitate. Cork the tube and put it away in the dark. One to three days later the contents of the tube will be found to have converted themselves into a homogeneous brown jelly. Acidified osmium is always said to be a precipitant of albumin, but I have performed this experiment repeatedly, and always got the same result. Add 10 c.c. of distilled water. The jelly does not dissolve. This experiment constitutes a strong recommendation of acidified osmium as a cytoplasm fixative.

The effect of acidified osmium on nucleoprotein is

wholly different. There is an immediate rather coarse precipitate. Acidified osmium can therefore not be recommended as a fixative for the interkinetic nucleus.

Fischer says that albumins are precipitated from acid solutions, and that 1% osmium will not precipitate nuclein nor nucleic acid under any circumstances, whether acidified or not. I can only suppose that these results were obtained by using great excess of albumin, under quite different conditions from those of ordinary fixation. Berg also never got a precipitate with nuclein, using 2% or weaker. Fischer considered tissues as quite unfixed by osmium (and also by unacidified potassium dichromate). He regarded fixation as taking place on subsequent treatment with alcohol during dehydration. In this he was certainly wrong. Osmium is particularly useful because it *prevents* the precipitating effect of alcohol during dehydration.

What happens during osmium fixation is not exactly known. Brownian movement immediately ceases in albumin solutions. Berg (1927) thinks that there is a primary and a secondary effect. The former involves the combination of the whole molecule with the amino-groups of the proteins. This primary fixation does not prevent staining, and at this stage cells may still be stained in the presence of osmium. The secondary effect is an oxidation of the compound formed, during which a part of the osmium tetroxide which has not already combined is reduced to a lower oxide or hydroxide, which makes the tissues brown or black. After the oxidation the tissues are only stainable after thorough washing. Pyrogallol or other reducers may be added to hasten the secondary effect. The instantaneous prevention of precipitation by alcohol is presumably part of the primary effect, and the gradual setting of egg-white to a jelly is part of the secondary effect.

Osmium is the only fixative which fixes both fats and conjugated lipides. Fats are blackened. This is

because all fats in animal cells contain some olein, and olein is an unsaturated substance which reduces the osmium to the blackish lower oxide or hydroxide. The fat is itself rendered insoluble in most fat solvents. It remains slightly soluble, however, in xylene, though not in benzene (Berg, 1927), while it dissolves readily in turpentine, probably because this oxidizes the osmium and thus undoes the fixation. Pure palmitin and stearin are not blackened by osmium.

Conjugated lipides do not reduce osmium tetroxide so energetically as fats, and hence usually appear slightly or not at all darkened. In the presence of potassium dichromate or chromic acid they do not reduce osmium at all, and therefore black globules seen in Flemming (p. 67) or Altmann (p. 69) material are probably fat and not conjugated lipides (Gatenby and Cowdry, 1928). Although not blackened, they are preserved by mixtures of chromic acid and osmium tetroxide. Since the former alone does not preserve them, it is clear that the action of osmium is not simply an oxidation. Osmium is a splendid fixative for mitochondria. Kopsch discovered in 1902 that prolonged soaking in osmium solutions blackened the Golgi element. (See p. 71.) This is regarded as evidence of its lipide nature. The best method for showing the Golgi element depends on this reaction.

The great disadvantage of osmium is its uneven fixation. The outermost cells of a block of tissue are 'overfixed' and rendered opaque and difficult to stain. Next comes a thin layer of beautifully fixed cells, and inside that all is unfixed by the osmium, but fixed by the other components of the mixture, if a mixture is used. If the other components do not dissolve fat, the osmium will come along after them and fix it: but the general fixation will be that of the other components.

Tellyesniczky found that $\frac{1}{2}\%$ osmium tetroxide penetrates only 1–$1\frac{1}{2}$ mm. in twelve hours. Underhill found with liver an enormously higher rate

(0·27 mm. in fifteen minutes), about the same as that of picric acids and chromic acids ; but I cannot think that this is a usual speed for most tissues. Long fixation (a day or more) is advisable with osmium on account of its slow action on proteins. It is claimed that the vapour of osmium tetroxide penetrates more rapidly than solutions. This seems almost incredible, and could only be believed if an objective proof were given. When the vapour reaches the outside of the piece of tissue, it can only penetrate further by dissolving in the fluids of the tissue. It is then no longer a vapour. How can its being a vapour *outside* affect the penetration of its solution *inside* ? Nevertheless, it may very well be used as a vapour, as Cramer suggests (1919) : nothing can then be dissolved out of the cells during fixation.

Wetzel found little hardening during thirty-six hours' fixation, his figure for resistance to bending being only about 170. The fact that alcohol does not precipitate after osmium fixation is emphasized by the fact that the figure is actually *reduced* after four days' subsequent treatment with 80% alcohol.

Kaiserling and Germer found slight swelling of cows' eggs placed in 1% osmium solutions after saline (from 171 μ to 172 μ, from 139 μ to 142 μ, and from 149 μ to 161 μ). Osmium does not shrink cells, but sometimes they shrink during the after-treatment, and there is a tendency for tissues to crack across. The consistency is often rather crumbly, especially after very prolonged fixation, and the tissue sometimes falls to pieces in melted paraffin or during the cutting of the sections.

Fig. 2 shows the splendid fixation given by 1% osmium tetroxide. The cells are far more life-like than those fixed in any of the other single fixatives. Strangeways and Canti found almost perfect preservation with a 2% solution, but it must be remembered that there was no after-treatment in their case. Mitochondria were unchanged, and there was scarcely

any precipitation in nucleus or cytoplasm. Spindle-fibres were not seen in dividing cells. Since these structures are not visible during life, even with the most painstaking illumination, and since they are not visible after fixation in fixatives which are not protein precipitants (osmium tetroxide and potassium dichromate), and since further they are extremely evident after fixation in powerful protein precipitants, it seems possible that they are artifacts. Micro-dissection studies give evidence in the same direction.

The salient points in this chapter are summarized below:

Alcohol. Reducer: incompatible with chromic, dichromate and osmium. Energetic precipitant of proteins, but nucleoprotein precipitate soluble in water. Denatures without combination. Lipides dissolved: glycogen precipitated but not fixed. Rapid penetration: great shrinkage and hardening. Staining difficult.

Formaldehyde. Reducer: incompatible with chromic, dichromate and osmium. Non-precipitant of proteins: makes additive compounds. Fixes conjugated lipides, hence mitochondria and Golgi bodies. Does not shrink, but often some precipitation and hence shrinkage in after-treatment. Hardens greatly (presumably cell-membranes).

Acetic acid leaves proteins of cytoplasm untouched. Precipitates nucleoproteins: is ideal fixative for chromosomes. Often destroys mitochondria. Swells tissues and leaves them absolutely soft.

Picric acid precipitates all proteins, forming protein picrates. Shrinks strongly: scarcely hardens.

Chromic acid. Strong oxidizer: incompatible with alcohol and formaldehyde. Precipitates all proteins. Must be thoroughly washed out. Facilitates staining with basic dyes.

Potassium dichromate. Oxidizer: incompatible with alcohol and formaldehyde. Fixes homogeneously without precipitating: dissolves chromatin,

Fixes conjugated lipides, hence mitochondria. Must be thoroughly washed out. When acidified precipitates proteins.

Mercuric chloride precipitates all proteins without combining with them : precipitates soluble in potassium iodide. Penetrates rapidly : hardens greatly. Must be washed out with iodine. Facilitates staining.

Cobalt nitrate facilitates subsequent impregnation of Golgi bodies with silver.

Osmium tetroxide. Strong oxidizer : incompatible with alcohol and formaldehyde. Fixes homogeneously without precipitating. Fixes and blackens fat and Golgi bodies : fixes mitochondria. Fixes very unevenly at different depths. Does not shrink at all : often shrinkage and crumbling in subsequent treatment. Must be carefully washed out. Staining difficult.

CHAPTER IV

FIXING MIXTURES

THE reader will realize that each of the various fixatives described in the last chapter has its own advantages and disadvantages. In order to achieve better fixation, it is natural that people should have tried mixing them in various ways. The measures given below are volumes, except where the contrary is stated.

CARNOY'S FLUID

(for chromosomes and glycogen)

Ethyl alcohol, absolute	3
Acetic acid, pure	1

This fluid was introduced by J. B. Carnoy in 1886. Carnoy was Professor of Cellular Biology (or General Cytology) in the Catholic University of Louvain. It appears that he was also a canon. He used it for the study of the oogenesis of the Nematode Spiroptera strumosa. He gives no indication of having tried other proportions in arriving at the 3 : 1 formula, but later in the same year he gave another formula including chloroform, which is much quoted, but which in my hands has given results inferior to the first. This much-quoted formula (alcohol 6, acetic acid 1, chloroform 3) is given in small print as a third footnote. It should only be tried in cases where penetration is particularly difficult, for instance, with eggs provided with tough envelopes.

Carnoy's fluid is a good fixative for chromosomes.

It dissolves lipides and hence the Golgi element and usually the mitochondria, but it precipitates glycogen. It penetrates very rapidly and so can be used with quite large pieces. Half an hour suffices for the fixation of small pieces. The outermost cells of a piece of tissue are distorted in the same way as was described under ethyl alcohol. The fluid is a rational one, for the acetic acid prevents the shrinkage and extreme hardening which would be caused by the alcohol. The alcohol fixes the cytoplasm and the acetic acid the nucleoproteins. The acetic acid should be washed out with several changes of absolute alcohol. If it is specially desired to preserve glycogen, the tissue may be left twenty-four hours in absolute alcohol.

Bouin's Fluid

(for chromosomes)

Formaldehyde, 40%	25
Acetic acid, pure	5
Picric acid, saturated aqueous	75

Osmotic pressure : about 100 *atmospheres*

Bouin elaborated his famous fluid at Nancy in 1897. It is probably more widely used than any other fixative. Bouin gives no record of experiments with different proportions : he says simply, '. . . je me suis bien trouvé de la combinaison suivante ', and then proceeds to give the formula in the proportion 10 : 2 : 30. Many cytologists must have been slightly worried that the figures, as usually given, add up to 105 and not 100. Bouin used his fixative for his important study of the changes caused in the mammalian testis by cutting the vas deferens, which led to his conclusion that the interstitial cells produce the male hormone.

Bouin's fluid, besides being very useful for general histological work, is an excellent fixative for chromosomes. Mitochondria are not usually fixed. The fluid penetrates quickly and fixes evenly. Small

pieces are often fixed for only half an hour, but I prefer several hours or a day, so as to give the formaldehyde time to exert its full effect. Tissues suffer no damage by remaining weeks in it. This makes it very convenient for work in the field, as apparatus for dehydration and embedding may be left at home. It keeps indefinitely, despite Bouin's own recommendation that it should be made up just before use. The chromosomes are no doubt fixed mainly by the acetic, the formaldehyde probably restrains the too coarse precipitation of the cytoplasm by the picric and of the nucleoproteins by the acetic, while the picric gives a sufficiently soft consistency for easy sectioning and makes staining easy. No special washing out is required, the tissue being simply dehydrated from 50% or 70% alcohol. (The yellow colour may be removed, if desired, as explained under the heading of picric acid.) With all these advantages, there is no wonder that Bouin is popular. It must be mentioned, however, that Carleton finds 'atrocious' fixation of kidney (1926).

Allen (1916) tried a number of modifications of Bouin's fluid, in an attempt to prevent clumping of metaphase chromosomes and shrinkage of the cytoplasm. Acting on McClung's suggestion he claims to have improved the fixation considerably, and his fluid should be tried in chromosome studies, if after ordinary Bouin they are hard to count. Allen worked with the brain and testis of the mouse. To 105 c.c. of Bouin he adds 1·5 gram of chromic acid. When this has dissolved, he adds 2 grams of urea. A precipitate forms if the urea is added too soon (Allen, 1918). Miss Carothers (1917), in her striking cytological proof of the segregation of the chromosomes in Orthoptera, used a 15 : 10 : 75 Bouin, with the addition of ½% of urea, for the somatic chromosomes of the female. Nakahara (1919) found no advantage in the addition of urea in his work on the spermatogenesis of the stonefly.

Goodrich (1919 and personal communication) finds that the use of iodine with Bouin prevents the shrinkage of the delicate cell processes of invertebrate leucocytes. Tissues may be fixed for a short time in a sherry-coloured solution of iodine in potassium iodide solution, and then transferred to Bouin, or they may be fixed in Bouin tinged brown by the addition of the iodine solution.

Flemming's Fluid

(for chromosomes, mitochondria and fat)

Chromic acid, 1%	.	15
Osmium tetroxide, 2%.		4
Acetic acid, pure .	.	1 or less (to be added just before fixation)

Osmotic pressure with full acetic : 23 *atmospheres.*
Osmotic pressure with only five drops acetic : 4·3 *atmospheres*

Walther Flemming, Professor of Anatomy at Kiel, published his great book *Zellsubstanz, Kern und Zelltheilung*, in 1882. He tells us how he based his fluid upon von Flesch's mixtures of chromic acid and osmium tetroxide. He found that the addition of acetic acid made fixation quicker and nuclear figures sharper, while staining was improved. He tried omitting the chromic, with unfavourable results. The first figure of a cell fixed with Flemming's fluid is an epithelial cell from the salamander. Two years later (1884) he greatly improved his fluid by making it very much stronger and changing the proportions. The strong fluid is the one given at the head of this section. It is wrongly quoted in every book on technique with which I am acquainted. Flemming said distinctly : 'Eisessig : 1 Maasstheil oder weniger.' 'Or less' is always omitted from the formula, to its detriment. It seems worth mentioning that Flemming used to fix for a day or more, wash for an hour or longer, harden in absolute alcohol, and then cut his sections with a hand razor under alcohol.

He elaborated his second fluid for the study of cell-division and nucleoli.

This fluid, with the full amount of acetic, is an excellent fixative for chromosomes. Its great disadvantage is the unevenness of its fixation. The outermost cells of a piece of tissue are generally overfixed by the osmium (p. 60), while the interior is fixed by the chromic and acetic alone so far as the proteins are concerned, the osmium arriving later and fixing the fat. In between is a layer, often only a few cells thick, of splendidly fixed cells. One day's fixation is usually best. Some workers give only an hour, but it is unlikely that the osmium will have completed its work in that time. The osmium gives a homogeneous fixation of the cytoplasm, prevents too crude a precipitation of the nucleoproteins of the interkinetic nucleus, and preserves and blackens fat, while the acetic and chromic preserve the chromosomes admirably. The chromic further gives sufficiently firm consistency and facilitates the staining of the chromosomes with basic dyes. Mitochondria do not usually show after Flemming with full acetic, though McClung finds almost perfect preservation of them in Orthopteran spermatocytes if the washing out of the fixative and the dehydration are made as short as possible (1929). It appears that if the fixative is not too thoroughly washed out, they do not dissolve quickly in alcohol. Ordinarily, for chromosome studies, Flemming should be washed out for twelve or twenty-four hours in running water.

Benda (1902) was the first to suggest extreme reduction of the acetic for the fixation of mitochondria. He recommends only three drops. Duesberg (1910) recommends five drops. Meves (1908) recommends three or four drops, but he also uses $\frac{1}{2}\%$ instead of 1% chromic acid, and dissolves the chromic acid in 1% sodium chloride solution instead of water. He claims thus to get less shrinkage with chick embryos. I have tested the weaker chromic both in water

and in sodium chloride with salamander cells, but the results were indistinguishable from those with the standard fluid with 1% chromic in water. Gatenby (1917 A and B) omits the acetic altogether. His splendid work on the mitochondria in the spermatogenesis of invertebrates was done with no acetic. Personally I have found shrinkage with this fluid, and I think that perhaps he may have exaggerated the harmful effect of acetic acid. Chickering (1927), working with the testes of Hemiptera, got only mediocre results with Gatenby's modification, but the addition of a few drops of acetic acid gave good results.

Flemming with reduced acetic is the ideal fixative for studying the mitochondria in spermatogenesis, because the chromosomes are reasonably well fixed by the acetic and chromic acids, and it is therefore possible to determine the exact stage in maturation of each cell, which is not possible with Altmann's fluid. (See below.) It is, of course, the osmium that fixes the lipides of the mitochondria. The acetic to some extent prevents shrinkage. Fixation may conveniently last for four days or a week. Duesberg (1910) finds Benda's complicated after-treatment unhelpful, but I think that three days' subsequent simple postchroming in 3% potassium dichromate often makes the fixation of the mitochondria more certain, at some risk to the chromatin. The tissue, whether postchromed or not, should be washed in running water overnight.

Altmann's Fluid

(for mitochondria and fat)

Potassium dichromate, 5%	1
Osmium tetroxide, 2%.	1

Osmotic pressure : 5·8 atmospheres

Altmann gave the formula for his fluid in 1890 in his book, *Die Elementarorganismen.* He was really

the discoverer of mitochondria, though he was mistaken about their nature and it was Benda who named them. He designed this fluid to show them. He remarks that while various fixatives show them in certain cases, his fixative is reliable with different classes of animals and different sorts of cells. He says that it penetrates better than osmium alone, and that although, like all osmium solutions, it makes staining rather difficult, yet good results are obtained with very thin sections.

The formula shows it to be a splendid reagent for homogeneous fixation of the cytoplasm, and it is probably the most certain of all fixatives for mitochondria. Chromatin, however, is dissolved by the dichromate, so it is useless for chromosomes and karyosomes. Its great disadvantage is that it leaves tissues in a state in which they tend to undergo shrinkage and crumbling during after-treatment. The sections often have cracks across them. Mr. Michael Thomas and I (unpublished results) have sought to improve this by using a weaker fluid. We have found equal parts of 3% dichromate, 2% osmium, and water a convenient fixative for the most varied tissues. We have found this suitable not only for mitochondria but also, with postosmification, for Golgi bodies. (See Mann's fluid.)

Altmann used to fix very small pieces for one day and then wash out, but I think it is best either to fix for four days or to fix for one and postchrome for three in 3% dichromate. The tissue should then be washed in running water overnight.

Champy (1911) modified Altmann's fluid by taking 1% chromic acid, 3% dichromate, and 2% osmium in the proportion 7 : 7 : 4. His fluid has been enormously used, both for mitochondria and Golgi bodies, but it seems preferable not to acidify dichromate solutions, for reasons which we have discussed. For showing Golgi bodies, I prefer generally to use Mann's fluid. Nevertheless, it must be

admitted that Champy's fluid has given excellent results in the hands of many workers. I have myself often found great shrinkage with it.

Mann's Fluid

(for Golgi bodies)

Mercuric chloride, sat. sol. in 0·75% sodium chloride	2
Osmium tetroxide, 2%	1
Distilled water	1

Osmotic pressure : 7·3 atmospheres

Mann (1894) designed his fluid for the study of nerve cells. He found mercuric chloride a particularly useful reagent for nerve cells. Adopting Heidenhain's solution in saline, he mixed it with osmium tetroxide, and thus obtained an excellent fluid which gives a life-like fixation of many sorts of cells. The osmium makes the fixation homogeneous and preserves lipides, while the mercuric chloride aids penetration, gives a sufficiently hard consistency to withstand after-treatment, and facilitates staining. Small pieces should be used.

In 1894, when Mann published his formula, Golgi bodies were unknown. In 1902 Kopsch discovered that the Golgi bodies of spinal ganglia were preserved and blackened by prolonged fixation (about eight days) in 2% osmium tetroxide. He worked with the rabbit, cavy, pigeon, duck, chicken, tortoise and frog. Osmium alone is not a satisfactory fixative, and in 1910 Weigl very much improved Kopsch's technique by fixing in Mann's fluid and then post-osmifying. He had parts of the central nervous system and mantle ganglia of Octopus, Sepia and Loligo fixed in Mann for a few to twenty-four hours, and sent to him by post in distilled water. On arrival he left them for many days in osmium solutions. Ludford (1924 and 1926) has made a special study of the Weigl technique for Golgi bodies, and improved it considerably. He has worked largely with tumour

cells. He fixes for from four to twenty-two hours in Mann, finding that the exact length of fixation is of no importance. He then washes for half an hour in distilled water. He carefully investigated the effect of the temperature on the blackening of the Golgi bodies, and found that three to five days in 2% osmium at 30° C., followed by one day in distilled water at the same temperature, gave the best results. The Golgi element is not impregnated at all at 0° and only slightly at room temperature. At 56° C. the whole cytoplasm is black. Incubators at 30° C. are not always available, and I find that 37° gives good results. After this postosmification the tissue may be washed for several hours in running tap water. No treatment with iodine is necessary. For some unexplained reason one does not appear to be troubled with the precipitates which are usual after mercury fixation. This method does not work over large areas of tissue, like the silver method, but it probably impregnates more precisely.

We have already discussed the propriety of dissolving mercuric chloride in saline solutions. Mann was fully aware of the solubility of mercury precipitates in saline. It is not possible to guess whether he used saline as a solvent in order to get a higher concentration of mercuric chloride, or to restrain its precipitating action. Its osmotic effect would be negligible. We have seen that a saturated solution of mercuric chloride in weak saline does precipitate. I have got good impregnations of the Golgi bodies in spinal ganglia using a saturated solution in water instead of saline when making up Mann's fluid. With epididymis I have found better general fixation with saline. The saturated solution is best made in boiling saline.

Mann says that his fluid should be made up immediately before use, but I doubt whether it deteriorates.

Da Fano's Fluid

(for Golgi bodies)

Formaldehyde, 40%	6–15 c.c.
Water	100 c.c.
Cobalt nitrate	1 gram

Osmotic pressure with 15 *c.c. of formaldehyde* 40% : 41 *atmospheres*

In 1898 Golgi discovered the bodies which bear his name by fixing nerve cells in a fixative similar to Altmann's and treating them with silver nitrate. The Golgi bodies slowly reduced the silver nitrate to silver and thus became blackened. In 1903 Cajal (see Bowen, 1928) published his 'photographic' method, which involved fixation in silver nitrate and reduction in a photographic reducer, hydroquinone. In 1907 he improved on this by fixation in formaldehyde, followed by silver impregnation and then reduction. In 1912 he discovered that the addition of certain nitrates to the fixative was favourable to the subsequent impregnation of the Golgi bodies with silver. He found uranium nitrate the best, but in 1920, as we have seen, da Fano reinvestigated the matter and recommended cobalt as giving more constant results (1920 A and C). The use of formaldehyde as a fixative is natural, since it fixes conjugated lipides, but the effect of cobalt is unexplained.

Fix for six to ten hours. The time of fixation affects the subsequent impregnation, so the optimum must be discovered experimentally. Rinse quickly twice in distilled water. Leave one or two days in 1·5% silver nitrate, away from the light. (Avoid staining the fingers.) Again rinse quickly in distilled water, and transfer to Cajal's reducer :

Hydroquinone	1·5 gram
Sodium sulphite	0·2 gram
Water	100 c.c.
Formaldehyde, 40%	6 c.c.

The formaldehyde presumably continues the fixation. It seems reasonable to use neutralized formalde-

hyde, for, in photography, hydroquinone will not reduce silver bromide in acid solution. The sulphite is itself a reducing agent, but has no power of reducing silver salts. It is added to protect the hydroquinone from being oxidized before use. If one uses acid formaldehyde, one is relying on the alkalinity of the sulphite to neutralize it.

Da Fano recommends about twelve hours' reduction, but Carleton (1919) finds it already complete in two.

Golgi bodies appear black, while the cytoplasm is rather an opaque yellow unless the section is subsequently toned (p. 80).

CHAPTER V

EMBEDDING

AFTER fixation every trace of water must be removed from the tissue, so that it may be impregnated with paraffin. This may be done by soaking in turn in 30%, 50%, 70%, 90% and two lots of absolute ethyl alcohol. (Certain advantages have been claimed for isopropyl and butyl alcohols. See Margolena, 1932, and Hatherly, 1932.) Gradual dehydration causes less shrinkage, or more even shrinkage, than direct immersion in absolute alcohol. If extremely gradual dehydration is thought necessary, one may adopt Allen's method of adding alcohol drop by drop (1916). With pieces of tissue 2 mm. or less thick, which is a usual size for cytological work, one hour's immersion in each of the strengths mentioned above is ample. For some inscrutable reason, Germans usually give a day in each. Half an hour in each of the two lots of absolute alcohol suffices. The shorter the time in the higher grades of alcohol, the less hardening and the easier it is to cut sections.

Alcohol and paraffin will not mix, so some other substance must be used as a go-between, which will mix with both. Benzene and cedarwood oil are the two dealcoholizing agents which I recommend. Xylene has been much used, but undoubtedly shrinks and hardens more than either of these (Romeis, 1928, and Tarkhan, 1931). Dealcoholization is often called ' clearing ', because it results in translucency, but the word gives the false impression that some change takes

place in the tissue itself. It is simply that the tissues have a high refractive index, and so have the dealcoholizing agents. Accordingly there is little reflection of light at the surfaces of the tissue when it has been thoroughly permeated. Light passes straight through and gives translucency. This is an indication that dealcoholization is complete.

Benzene, C_6H_6, was discovered by Faraday in 1825, and its structural formula, a closed ring, was given by Kekule forty years later. When tar is distilled, the distillate is collected in vessels which contain water. Part of the distillate, called the light oil, floats. This is treated with sodium hydroxide to remove the phenol it contains, and with sulphuric acid to remove basic substances. It is then fractionally distilled. The first distillate, coming off between 80° and 100° C., is mainly benzene. It is cooled to 0° C., when the benzene crystallizes and the impurities are poured off.

Benzol is a synonym for impure benzene, but should be avoided, as the -ol termination suggests an alcohol or phenol. For the same reason xylene should not be called xylol. Benzine has nothing to do with benzene. It is a trade name given to a mixture of paraffins.

It is best to transfer tissues from absolute alcohol to equal parts of alcohol and benzene (half-hour), from that to pure benzene (half-hour), from that to a warm solution of solid paraffin in benzene (quarter-hour), and from that to pure melted paraffin (one hour), which should be changed once or twice. The times given are ample for small pieces. The paraffin is then cooled suddenly with water, so that it may set homogeneously. For full instructions, see Carleton's book (1926). Paraffin of melting-point 50°–55° C. is used. It is easier to cut very thin sections with high melting-point paraffin, but the sections do not form ribbons so easily. The paraffin oven must be kept as cool as is consistent with its not solidifying. Great shrinkage occurs in hot paraffin (Tarkhan, 1931).

It is possible to pass from 96% alcohol to benzene, without passing through absolute alcohol at all, if phenol is dissolved at 3% to 5% in both the 96% alcohol and the benzene. Shrinkage and the high cost of absolute alcohol are thus avoided. (See Barta, 1923, and Bacsich, 1932.) In the absence of phenol, a cloudy emulsion of water in benzene is formed when 96% alcohol and benzene are mixed. I prefer, however, to avoid absolute alcohol by using cedarwood oil instead of benzene. Cedarwood oil shrinks and hardens less than any other dealcoholizing agent, and tissues may be left indefinitely in it, if it is inconvenient to embed it once. These are tremendous advantages. It has the disadvantages of being messy, because it does not evaporate completely like benzene, and also is rather expensive.

Cedarwood oil is a thick pale-yellow essential oil derived from the Virginian Juniper, *Juniperus virginiana*. Juniperus is a genus of the pine family, allied especially to the Cypress and Thuja. The Common Juniper is generally only a shrub not higher than a man, from the unripe fruit of which oil of juniper is obtained. This oil is used for flavouring gin, and is not to be confused with cedarwood oil, which is derived from the wood of *J. virginiana*. This tree, often improperly called the Red Cedar, grows in North America, where it attains a height of about 40 feet. The term cedar is obviously inappropriate, for the juniper has a fruit of only three carpels, while the cedar has a cone of many. Further, the leaves of the juniper are very small and overlap one another like scales. The wood of the Virginian Juniper provides the 'cedar' of cigar boxes and lead pencils. The oil is prepared by distillation of the shavings made in the manufacture of the pencils (Parry, 1918). It is used in perfumery. It is an essential oil, consisting of a sesquiterpene ($C_{15}H_{24}$) called cedrene, and cedrene camphor ($C_{15}H_{26}O$), which is a crystalline solid. In essential oils it is usual to find terpenes

and oxidation products of them. The pleasant smell is mostly derived from the cedrene. This, being volatile, may be evaporated off, and the thickened oil is used as immersion oil in microscopy, on account of its refractive index and dispersion being nearly the same as those of glass.

Cedarwood oil is stated to mix with 95% alcohol, but I find that it will not mix even with 97% alcohol except in certain proportions. Nine parts of cedarwood oil with one of 97% alcohol form a clear solution, and so does one part of oil with nine of alcohol. However, when eight parts of either are shaken with two of the other, mixture does not take place, and a solution of oil in alcohol above remains separate from a solution of alcohol in oil below. This makes one chary of transferring tissues from anything but absolute alcohol to pure cedarwood oil, and indeed it sometimes occurs that it simply will not dealcoholize unless absolute alcohol has been used. I therefore transfer tissues from 97% alcohol to a mixture of absolute alcohol and cedarwood oil in equal parts for about half an hour (for small pieces), and from that to pure cedarwood oil for half an hour, or for any greater period that may be convenient, even for months.

Cedarwood oil is not so easily replaced by paraffin as benzene. To overcome this difficulty, I transfer tissues from cedarwood oil to a warm solution of paraffin in equal parts of cedarwood oil and benzene for half an hour, in a covered capsule on top of the embedding bath. The mixture of cedarwood oil and benzene is a much better solvent for paraffin than cedarwood oil alone. This paraffin solution may be used again and again. From this the tissue is transferred to pure paraffin, where it remains for about an hour, being renewed twice during that period. I almost always use this technique when embedding, and can strongly recommend it.

Romeis (1928) says that cedarwood oil retains the black of reduced osmium better than any other

dealcoholizing agent. It should therefore be especially useful in studies of fat and Golgi bodies.

Embedding in celloidin does not permit of the cutting of very thin sections and it will not be described here, though in the hands of some workers it gives splendid results.

The methods of cutting sections and attaching them to slides are excellently described in Carleton's *Histological Technique* (1926). For general cytological work 5 μ or 6 μ is a convenient thickness. 2 μ or 3 μ is advisable with Altmann's method for mitochondria, and up to 20 μ may sometimes be necessary in chromosome work, to ensure getting all the chromosomes in an equatorial plate in the same section.

The tissues of the section are impregnated all through with paraffin, and this must be removed before they can be stained. Xylene is a most convenient solvent. Commercial xylene is mostly meta-

xylene, , with smaller amounts of ortho- and paraxylene. Like benzene, it is a component of coal tar. The slide is left in xylene for two minutes, or longer if the section is very thick. Two minutes' immersion in absolute alcohol suffices to remove the xylene. There is now no point in avoiding absolute alcohol, because hardening does not matter after sectioning, and shrinkage does not occur when the section is stuck to the slide. After a quarter of a minute in 90% alcohol and the same in 70%, the slide is ready for staining in a dye dissolved in alcohol. If the dye is dissolved in water, it may be transferred direct from 70% alcohol to distilled water and left for a minute. All the times given in this paragraph may be extended indefinitely, but no advantage is gained.

If the Golgi bodies have been impregnated with

osmium or silver, it is often best not to stain any other part. The slide is then mounted (see p. 117) directly the paraffin has been removed by xylene.

After silver impregnation the silver may be replaced by gold. The principle of this replacement was introduced into photography so long ago as 1850. In cytology it has the advantages that it renders the ground cytoplasm transparent and very pale grey instead of rather opaque and yellowish, and that the preparations are rendered permanent. Da Fano's technique for gold-toning is as follows (1920 B). (His first description (1919) contains a serious misprint.) Bring the slide to distilled water, and transfer it to 0·1% gold chloride solution. Gold chloride forms yellowish orange crystals. In a minute or so this yellow solution has removed the yellow colour from the sections. When microscopical examination shows that this has happened, rinse the slide with distilled water and transfer it to 5% sodium thiosulphate ($Na_2S_2O_3$, 'hypo'). This removes any unreduced silver which may still remain, and makes the section more transparent. Then wash in a stream of distilled water. I have found thionin (p. 106) a convenient nuclear stain, but one may mount (p. 117) direct without staining if one prefers.

One drop of pure acetic acid may be added to every 10 c.c. of the gold chloride solution. This appears to hasten the replacement, and results in a faintly purple background.

CHAPTER VI

THE PRINCIPLES OF STAINING

'The method of staining, once having taken root in the animal histologist, grew and grew, till to be an histologist became practically synonymous with being a dyer, with this difference, that the professional dyer knew what he was about, while the histologist with few exceptions did not know, nor does he to the present day.'—(MANN, 1902.)

IT is possible to make out a fair amount of structure in cells without staining them, on account of the different parts having different refractive indices. One can see chromosomes and other cell structures just as one sees a colourless glass rod held under water. Parts of the cell that have the same refractive index as their surroundings are invisible, for the same reason that a clean glass rod is not easily seen when in Canada balsam (p. 118). The advantage of staining, even when the refractive indices are different, are so great that it is almost universally practised, except when certain parts of the cell have already been rendered black by impregnation with osmium or silver before sectioning. One may either stain certain parts and leave the background unstained, or else one may stain different parts with different colours, relying on their different aptitudes for being stained with different sorts of dyes.

What are dyes, and what different sorts are there ? [1]

[1] I wish to acknowledge great help from Conn (1925), Heinrich (1922), Kipping and Kipping (1931), Knecht, Rawson and Lowenthal (1916), Perkin and Everest (1918), Remsen (1909), and Wahl (1914). I have followed Conn's nomenclature.

As a general rule, aliphatic compounds are not only colourless, but have no absorption bands in the ultra-violet. Benzene ring compounds, on the contrary, usually have absorption bands in the ultra-violet. Benzene itself has, although of course it is colourless. Certain chemical compositions shift the absorption bands of the ring compounds into the visible spectrum, and colour results.

One of the particular arrangements which confers colour is the *quinonoid* arrangement of the linkages in a ring compound. In an ordinary, non-quinonoid ring compound, the linkages may be expressed as shown below, though there is some doubt as to whether this really represents the actual state of affairs. Hydroquinone, the substance shown, is colourless :

(A simple hexagon, ⬡, will be used throughout the rest of this book for non-quinonoid rings.)

Now when hydroquinone loses two hydrogen atoms, by oxidation, its whole structure is changed. The quinonoid linkage makes its appearance, and the resulting substance, quinone, is yellow :

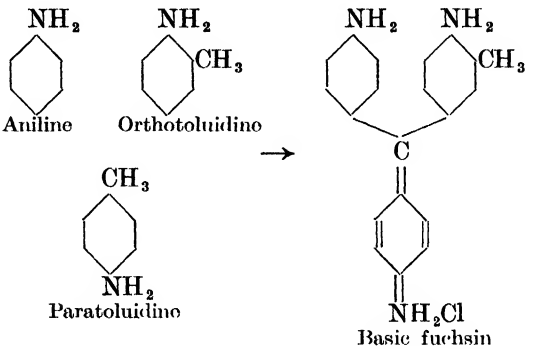
Nevertheless, quinone is not a dye. Fixed tissues allow the diffusion into them of all crystalloids, and

if the crystalloids are coloured, the tissue will be temporarily stained. In many cases, however, the colour will diffuse out again just as easily as it diffused in, and when the tissue is soaked in the solvent that was used for the colour, it is removed and the tissue left colourless. Such coloured substances are not dyes. A dye is a coloured substance which has a special aptitude for being retained by tissues. Such aptitude is conferred by the addition of certain radicles to a substance which already has the quinonoid linkage, or some other arrangement of atoms which confers colour. The most important of these radicles are NH_2 and OH. Every one has heard of aniline dyes, and it is the presence of the amino-group, NH_2, in aniline that makes it so important in their manufacture. Aniline itself has not got the quinonoid linkage, and it is a colourless fluid when pure. It is a poisonous substance, slightly soluble in water. Anil is the French for indigo, and it was from the indigo plant that aniline was first obtained, by distillation. It occurs as such in coal tar, but on the commercial scale it is made from benzene. Nitrobenzene is first prepared, by the action of nitric acid on benzene. This has two oxygen atoms in the place of the two hydrogen atoms of the NH_2 group of aniline. Nascent hydrogen, produced by the action of iron on hydrochloric acid, effects the substitution.

The toluidines are very similar to aniline, and are prepared in a comparable way. They are the same as aniline in chemical composition, except that they contain a methyl group as well as an amino group. The two that interest us are orthotoluidine and paratoluidine.

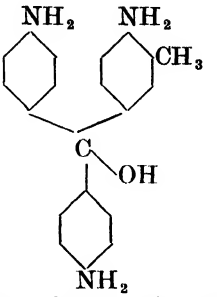
If some aniline, orthotoluidine and paratoluidine are mixed together in the proportion of one molecular weight of each, and if hydrogen is removed and chlorine added, the three benzene rings join together into one molecule. The ring which was formerly the paratoluidine ring assumes the quinonoid struc-

ture, and a splendid magenta dye, basic fuchsin, has been produced. The reaction may be represented as follows :



It is very easy to prepare a little basic fuchsin. Ordinary commercial aniline contains some ortho-toluidine and paratoluidine, and one may therefore use commercial aniline instead of the mixture in molecular proportions. The yield of basic fuchsin is naturally less, but the reaction occurs. One puts into a dry test tube ten drops of aniline, and adds as much powdered mercuric chloride as will lie on the last quarter of an inch of the point of a scalpel. The purpose of the mercuric chloride is to act as an oxidizing agent, that is, to remove the hydrogen. One now heats the tube and boils the aniline for about ten seconds. Then, while it is still hot, one adds about 10 c.c. of 70% alcohol, acidulated with five drops of hydrochloric acid. A magenta-coloured solution of basic fuchsin is produced. In commerce the oxidizing agent used is not mercuric chloride, but arsenic acid.

It will be noticed that basic fuchsin is a chloride, and many dyes are also chlorides. It is the chloride of a colourless substance called rosanilin. This has the composition



It is the absence of the quinonoid linkage that results in rosanilin being colourless. It is not the mere conversion into a chloride that causes colour to appear, for another chloride exists which is colourless, on account of there being no quinonoid linkage.

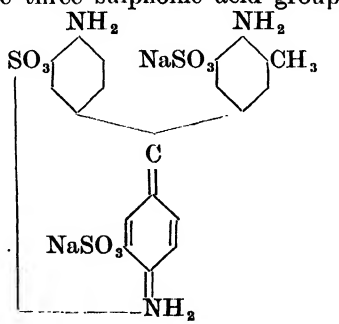
Dyes which are chlorides (or sulphates or acetates) of organic bases are called basic dyes, and basic fuchsin is so called for this reason. It was first used commercially in 1859, only four years after the discovery of the first aniline dye, mauve, by W. H. Perkin. It is used for dyeing cotton, wool and silk. In cytology it is used as an ordinary stain for chromatin, but its most important use is in Feulgen's microchemical test for nucleic acid. (See Ludford, 1928.)

Basic fuchsin has three NH_2 groups, and in allied dyes the hydrogen of some or all of these groups is replaced by methyl, CH_3. There are six hydrogen atoms capable of being replaced by methyl. The substitution of methyl for the hydrogen of amino groups results in change of colour from magenta to violet and from that to blueish violet. Thus crystal violet has six methyl groups.

Crystal violet is so called on account of the colour which it dyes and the large crystals which it forms with nine molecules of water. The crystals have rather a dirty greenish sheen, but they dissolve to form a beautiful blueish violet solution. This is another basic dye, used for cotton, wool and silk. In

biology its chief use is for the staining of mitochondria by Benda's technique. (See p. 102.) It must be mentioned that it differs from basic fuchsin not only in having six methyl groups attached to nitrogen atoms, but also in having no methyl group except those six. Basic fuchsin has one methyl group not attached to a nitrogen atom. Ethyl groups have still more blueing effect than methyl, and phenyl groups carry it farther still, till pure blue dyes are obtained. These are not used in the techniques described in this book, so they will not be described here : but it is important to understand the sort of way in which the chemist makes dyes of various colours.

The fact that certain dyes are called basic dyes suggests that there must be others called acid dyes. In acid dyes the dyeing radicle of the molecule is the anion, which is combined with a metal, usually sodium or potassium. An acid dye may be made by treating basic fuchsin, or its colourless base, with fuming sulphuric acid at 120° C. A sulphonic acid is thus obtained. A sulphonic acid is sulphuric acid in which an organic radicle has replaced a hydrogen and an oxygen atom. Thus if sulphuric acid is written as $HO.HSO_3$, it is easy to see how it may be converted to ethyl sulphonic acid, $C_2H_5.HSO_3$. The sulphonic acid of basic fuchsin has three sulphonic acid groups, one for each ring. The dye, acid fuchsin, is made from this acid by putting sodium in the place of hydrogen in two of the three sulphonic acid groups.



If sodium is introduced into the sulphonic acid attached to the third ring, the quinonoid structure is lost, and it is no longer a coloured substance.

Acid fuchsin is largely used for dyeing wool and silk. It will not dye vegetable fibres. It has the practical disadvantage of not being fast to light. In cytology its most important use is as a stain for mitochondria by Altmann's method, which is fully described on page 112. It can also be used as an ordinary cytoplasm stain, in $\frac{1}{2}\%$ solution in water. A few minutes suffice after many fixatives, but after Flemming's fluid a day may be necessary. Acid dyes are removed from tissues by alkalies, and so acid fuchsin may be partly removed by tap water if it has overstained, if the tap water is of ordinary alkalinity.

Another acid dye, far more complicated but of the same general chemical type as acid fuchsin, is light green SF. It is also a sodium sulphonate, with three sulphonic groups, but it differs from acid fuchsin in that one of the three rings has no nitrogen atom attached to it, while two more rings are indirectly attached to the other two rings. Light green is not very fast to sunlight, so it is not much used for dyeing clothes, but it finds its application in the dyeing of billiard cloths. In cytology its chief use is as a counter-stain to safranin. Safranin is a red dye, used for staining chromatin, and the redness shows up to the best advantage if the cytoplasm is extremely lightly stained in green. This technique is described on page 109.

The dyes so far described have three phenyl rings as an essential part of the molecule, and may be described as triphenyl dyes. The usual name is triphenylmethane, but the word is not without objection. Other triphenyl dyes commonly used in biology are:

Basic: Hoffman violet (= dahlia); gentian violet (a mixture of dyes); methyl green; spirit blue (= anilin blue).

Acid: methyl blue.

We have only discussed a few dyes, but we have studied them enough for it to be profitable for us to ask ourselves what the nature of the dyeing process really is. First of all, it has clearly nothing to do with painting, in which process insoluble pigments are attached to the surface of objects by sticky, partly volatile adhesives. In dyeing (or staining, for the words are synonymous) soluble dyes are caused to permeate and be retained by objects, without the use of adhesives. What causes them to be retained? Why are they not washed out again by the solvent?

No one knows. It is very surprising that no one should know, for we are surrounded by dyed objects. There are two main theories, the theory of chemical combination and the theory of adsorption. Let us take the chemical theory first, as it seems to me the most plausible so far as the dyeing of animal tissues is concerned. The dyeing of the vegetable fibres, cotton and linen, may be a different process.

When wool or silk is dyed with a solution in water of basic fuchsin, that is, of the hydrochloride of the colourless base rosanilin, the hydrochloric acid of the molecule remains in solution in the water, while the rosanilin is taken up by the fibre. Now the fibre is dyed magenta, by the *colourless* base rosaniline. What has happened? It is known that the salts of rosanilin are all magenta-coloured. Is it not extremely probable that something in the fibre, of acidic nature, has taken the place of the hydrochloric acid and formed with rosanilin a coloured salt? The facts speak strongly for chemical combination.

If we start with the colourless base rosanilin and boil some wool or silk in it, it is dyed magenta. Surely a chemical combination must have taken place, for there is no physical process known whereby rosanilin may be made into a coloured substance.

These arguments appear so conclusive, that one might fancy the matter clinched; but there are also arguments against the chemical theory. First, when

a chemical combination takes place, we expect to see a product quite unlike those we started with. Here, however, we start with wool and a substance of magenta colour, and we end with what is obviously wool, and of magenta colour. That does not look like a chemical combination. Nevertheless, we cannot say that it is not a chemical combination. If we treat cotton with a mixture of nitric and sulphuric acids, the product looks just like cotton; but it would be foolish and unfortunate if we concluded that no chemical combination had taken place, for in fact we would have in our hands a violent explosive. Another point in connexion with the dyeing of complex mixtures of organic substances such as wool, silk, or cytological material is that the dye may combine with only part of it, and the remainder may not unnaturally retain its properties. The same consideration must weigh with us when it is objected to the chemical theory that there is no definite proportion between the amounts of the substances reacting.

If the process were purely chemical, one might expect that in very dilute solutions no reaction would occur, that if the concentration were gradually increased a point would suddenly be reached at which the reaction would occur completely, and that no further increase in concentration beyond that would alter the result. This of course is not what happens in dyeing. Dye is taken up to some extent from surprising dilute solutions, and gradual increase in concentration results in gradual (though not directly proportional) increase in the amount of dye taken up.

Another argument against the chemical theory is that sometimes even sulphuric acid does not displace a dye whose acid radicle is sulphonic acid. One would certainly have expected it to do so, but it must be remembered that sulphonic acids are not necessarily weak, and are capable under certain circumstances of decomposing salts of sulphuric and

hydrochloric acid. Perhaps a stronger argument against the chemical theory is that no change of temperature is noted while dyeing is taking place.

It is the proportion between the concentration of the dye and the amount taken up by the object dyed that gives the strongest support to the physical theory. When the concentration is gradually increased, the amount of dye taken up is increased, but the increment is far less than proportional, and steadily falls as the concentration becomes higher. In other words, dyes stain proportionally more vigorously in weak solutions. This is similar to what happens in the physical process of adsorption.

Adsorption is the process of the attraction of one substance to the surface of another. It is called *ad*sorption because one substance is attracted *to* another and not *into* it. Finely divided material naturally shows the phenomenon in the highest degree, for the surface area is greatest. A jar full of powdered charcoal actually holds more air than an empty jar, on account of the adsorption of air on the surface of the carbon. Not only gases are adsorbed. Bacteria are readily adsorbed by charcoal. In the filters for bacteria, there is plenty of room for bacteria to pass between one particle of charcoal and the next, but they are held by adsorption. Whenever there is an interface between one substance and another, there is a possibility that adsorption may take place. The precipitated spongework of the ordinary fixed cell provides plenty of surface, and wool and silk are also porous.

The upholders of the adsorption theory of dyeing regard the process as partly one of electrical attraction. They explain the removal of acid dyes by alkalies on the assumption that OH ions are deposited on the tissue, which thus becomes negatively charged and repels the acid dye. Similarly, acids are supposed to remove basic dyes by depositing hydrogen ions. Alcohol is supposed to remove dyes because it reduces

the electrical charges which hold them to the surfaces of the tissue.

The fact that dyeing is relatively more intense from weak solutions is not necessarily opposed to the chemical theory, for it may be due to the dye molecule being more dissociated at great dilutions, and therefore more active chemically. A fact opposed to the adsorption theory of dyeing is that dyes act most strongly at high temperatures, although surface tension is decreased when the temperature is raised. The effect of heat supports the chemical view. Hot dyes are used in two methods for mitochondria (pp. 103 and 113).

According to another physical theory of staining, the dye actually dissolves in the substance stained. Substances certainly can dissolve in solids under certain circumstances, and fats can be stained by dyes that are soluble in them in this way. (See p. 112.) However, this is certainly not what happens in dyeing as a general rule. If solid solution occurred, the amount of the dye taken up would be directly proportional to its concentration in the dyeing bath, which is not so. Also the dye would be wholly removed by excess of the solvent used. Again, cellulose fibres are strongly stained by many dyes, but the same dyes fail to stain cellulose which has been rendered non-spongy by dissolving it in acetone and evaporating.

It seems possible that the dyeing of cotton and linen on the one hand, and of wool, silk and cytological material on the other, may be different processes, the former physical, the latter at least partly chemical. Cotton and linen consist of cellulose, which is rather an inactive substance chemically, neither basic nor acidic. The proteins of wool, silk and cytological material, on the contrary, possess both basic and acidic properties, and thus may be expected to combine with the colour radicles of acid and basic dyes. If this is so, there is no reason why physical adsorption should not occur at the same time, and indeed it seems probable that it does. If

the dyeing of cotton and linen is a different process from the dyeing of animal fibres and of proteins generally, then one would be surprised if the same dyes always acted on both : and indeed they do not. Most dyes do not act on vegetable fibres unless they have been treated with a substance called a mordant. Wool, silk, and proteins which have undergone fixation can be stained by most dyes without mordanting, but nevertheless mordanting is of extreme importance in cytology, for certain important dyes require it. Before we discuss mordanting, however, we must mention some differences between basic and acid dyes.

As a general rule, basic dyes are dyes for chromatin, and acid dyes are dyes for the cytoplasm. If we stain a section in a basic dye, say basic fuchsin, the chromatin and cytoplasm are both stained, but the chromatin is stained most deeply. We can now 'differentiate' ; that is, we can soak the section in a fluid which extracts the dye. Alcohol by itself often extracts basic dyes, particularly crystal violet, but if it is acidified by the addition of 1% of acetic acid, the extraction proceeds much more rapidly. This is probably because ordinary proteins, even when fixed, are still capable of behaving as bases in acid solutions and as acids in basic solutions. The cytoplasm of the cell, being rendered basic by the acid alcohol, has no longer any attraction for the basic dye, and it rejects it. The chromatin is so strongly acid that it is not at once rendered basic by the acid alcohol, and it retains the dye longer. If we wish, we may add the acid to the staining bath, and thus restrict the staining to such parts of the cell as have a particular attraction for basic dyes, that is, in most cells, the chromatin. We must realize clearly that we are not in any sense testing for chromatin microchemically, when we differentiate a basic dye and allow it to be retained only by certain cell-components ; we are only testing for substances which have a special attraction for basic

dyes. The Nissl substance in the cytoplasm of nerve ganglion cells is one of these. It retains basic dyes, but it is not chromatin.

When we have differentiated, and the cytoplasm is once more colourless, we may stain in an acid dye, such as light green. This will not affect the chromatin, unless it is allowed to act for too long, but it will stain the cytoplasm green. The acid dye is called counter-stain to the basic one. One should use a counter-stain of a colour which will cause the basic dye to show up clearly. Thus orange would be an unsuitable colour for a counter-stain to red. Complementary colours should be used if possible.

It must be stressed that although one usually stains the chromatin with basic dyes and the cytoplasm with acid ones, yet the basic dyes stain the cytoplasm as well as the chromatin, and some acid dyes (e.g. acid fuchsin) can be differentiated to give precise stains of chromatin. When this happens, they are probably combining with the protein part of the nucleoprotein molecule, whereas the basic dyes are regarded as attaching themselves to the nucleic acid. It is possible, however, that acid fuchsin may dissociate into sodium, sulphonic acid, and rosanilin, and that the latter basic substance may combine with the nucleinic acid to give a coloured salt.

Egg-white coagulated by heat has no affinity for either basic or acid dyes in neutral solution. An acidified solution of an acid dye will nevertheless dye it, and so will an alkaline solution of a basic dye. It is instructive to take two pieces of egg-white coagulated by absolute alcohol, and to put one into an alkaline solution of the basic dye thionin, and the other into an acid solution of the same stain (Mathews, 1898). If the two pieces are removed after a few seconds and washed in water, the first will be found to be stained, and the second scarcely tinged. The exact converse happens with an acid dye. The addition of alkalis to basic dyes and of acids to acid

dyes is therefore reasonable when for any reason one wishes to increase their action.

Certain substances increase the action of stains in a different way. Aniline is one of these. Aniline is only slightly soluble in water, but if a little is shaken up with water and the mixture filtered, the dilute aniline solution which passes through is a suitable medium in which to dissolve many stains, for it intensifies their action in an unexplained way. It appears that it may be regarded as a catalyst, if the chemical theory of staining be accepted. Such substances are called accentuators. Aniline is a basic substance, but it is used as an accentuator for both basic and acid dyes. For instance, it is sometimes used with safranine in staining chromosomes (p. 108), and with acid fuchsin for staining mitochondria (p. 112).

Other substances act in exactly the opposite way to accentuators, and are used to prevent overstaining. The use of acids with basic dyes for this purpose has been mentioned. Glycerol finds application here. Some dyes do not stain at all if dissolved in glycerol instead of water or alcohol, and the addition of glycerol to the staining bath results in slower and more even staining. It is used in Ehrlich's haematoxylin (p. 97). Alcohol is sometimes used with stains which are perfectly soluble in water, to restrain their action. The acid dye orange G. (p. 111) never overstains if used in saturated solution in absolute alcohol. As a general rule dyes are dissolved in water if they are sufficiently soluble in it and if no restraint is necessary. Dyes which are insufficiently soluble in water are nearly always dissolved in alcohol, generally diluted with water.

We now turn to the important matter of mordanting. A mordant is a substance which permeates the substance to be dyed and attaches itself to it, and subsequently combines chemically with a dye to form an insoluble coloured compound. For basic dyes an acid mordant is required, and cotton is usually dyed with basic dyes after mordanting with

tannic acid. Basic dyes are mostly used in cytology for staining chromatin, and for this purpose they require no mordant. When, however, a basic dye is used to stain mitochondria, as in Benda's technique (p. 103), an acid mordant is required.

In cytology mordants are chiefly used with certain acid dyes, and we shall therefore be concerned mostly with basic mordants. The most important of all dyes used in cytology is haematoxylin, and this requires a basic mordant. It will therefore be convenient to describe haematoxylin, as an introduction to the use of mordants. Haematoxylin is not a synthetic dye, but a vegetable product, and such dyes are often dealt with separately from the synthetic ones. This, however, does not appear necessary, for the staining principle of haematoxylin has a quinonoid linkage, and it is reasonable to consider it with the quinonoid synthetic dyes.

Haematoxylin

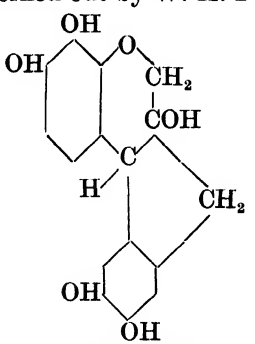
Haematoxylin deserves a full description (Bentley and Trimen, 1880, Rendle, 1925, and Perkin and Everest, 1918). It is derived from the wood of a smallish spreading tree with crooked branches, *Haematoxylon campechianum*, Linn. It is the only species of the genus. It is a native of Campeche, a state in Mexico, from which the specific name is derived. Its dyeing properties were known to the natives before the arrival of white people. The Spaniards brought the wood to Europe soon after the discovery of America. The tree was introduced into Jamaica in 1715, and it is largely cultivated there at the present day. It is a member of the family Leguminosae, or plants with pods. There are two sub-families in addition to that which includes our familiar broom, gorse, clover, and pea. The first sub-family includes the Mimosa and Acacia, which have regular flowers, quite unlike the zygomorphic (bilaterally symmetrical) flowers of the pea and its allies. The second sub-family is the Caesalpinioideae, which

includes Senna, the cathartic, and Haematoxylon. The Caesalpinioideae have zygomorphic flowers, but the sepals are nearly or quite separate, instead of being fused, as in the pea sub-family.

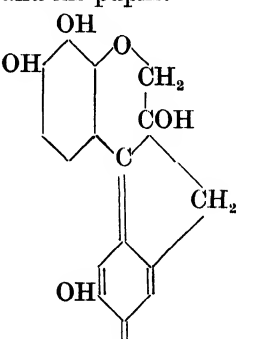
Haematoxylon campechianum has pinnate leaves, with four (or sometimes five) opposite leaflets on each. The pale yellow flowers are borne in racemes. The five petals only show slight zygomorphy, and the sepals are not quite separate from one another. The pod is remarkable because it does not split down the sutures, like a pea. Each valve splits down the middle of its length, disclosing one or two seeds.

The trunk of the tree is often much gnarled. The dyeing principle is contained in the heart-wood only. The tree is felled at the age of about ten years, and the bark and sap-wood is chipped off. The heart-wood is exported in 3-foot logs, whence the name 'logwood'. The logs are reduced to little chips for the extraction of the dyeing principle, which is called haematoxylin. Haematoxylin used to be used in medicine, especially as an astringent in infantile diarrhoea, and even for the treatment of cancer. Nowadays it is only used as a dye.

The structural formula for haematoxylin was worked out by W. H. Perkin and his pupils.



Haematoxylin



Haematein

It is not itself a dye. It requires first to be oxidized to haematein. I have rearranged the structural formulae somewhat to bring out clearly the quinonoid arrangement of one of the rings in haematein ($C_{16}H_{12}O_6$), but not in haematoxylin ($C_{16}H_{14}O_6$). The oxidation takes place gradually over a period of months of 'ripening'. It has recently been shown (Kohl and James, 1931) that exposure of solutions in evaporating dishes to the ultra-violet light of a strong mercury vapour lamp two feet away, with frequent stirrings, effects the oxidation in four hours or less. It might be thought more rational to use haematein itself in making up one's solutions, but haematein has a tendency to be further oxidized to a substance without staining power. It therefore seems best to use haematoxylin which has been partly ripened. One's solutions will then probably contain haematoxylin, haematein, and the further oxidation product. So long as they contain haematein, they will stain well. The exact amount of haematein present is not a matter of much moment.

Logwood extract was first used in cytology in 1862 by Waldeyer, the man who gave chromosomes their name. In 1886 Paul Ehrlich, who was later to become world-famous for his discovery of a cure for syphilis by means of organic arsenic compounds, published his formula for the dye which has probably been more used in histology than any other. The whole communication (1886) is in less than three dozen lines. It is in the form of an answer to a query by Bolles Lee, published in the previous volume of the same journal, asking for information about the haematoxylin solution which Ehrlich was using. Haematein is a weak acid dye which will scarcely stain tissues unless used with a mordant. Ehrlich used alum (potassium aluminium sulphate) as a basic mordant. Mordants may either be mixed with the dye, or used before it. Ehrlich mixed them. He says that it was already well known that such mixtures

give rise to a blue precipitate. Mann (1902) says that the precipitate is formed by the combination of haematein with $Al(OH)_3$. This hydroxide only forms in alkaline solutions. The alkalinity may be derived from the glass of the vessels in which it is kept. Ehrlich says he sought to prevent the dissociation of the alum by the addition of acids. The first attempt, with acetic acid, gave the desired result: precipitation was avoided. The acid also prevents the diffuse staining of the cytoplasm, so that it is chiefly the nuclei that are stained. We have already discussed the significance of the glycerol. The formula is as follows:

Water	100 c.c.
Absolute alcohol	100 c.c.
Glycerol	100 c.c.
Acetic acid (pure)	10 c.c.
Haematoxylin	2 grams
Potassium aluminium sulphate in excess.	

The mixture ripens in the light and becomes reddish. One may stain for about twenty minutes. Everything goes red, especially the nuclei, but the desired compound of haematoxylin with the basic mordant has not been formed, as it only forms in alkaline solutions. The slide is put in running tap water, which is alkaline. A blue compound of haematein with aluminium hydroxide is formed. A compound between an acid dye and a basic mordant (or rather the part of the mordant left in the tissue) is called a 'lake'. Examination with the microscope usually shows that the lake is rather diffusely distributed at first. The slide is placed for a few seconds in a staining jar of distilled water acidified by a few drops of hydrochloric acid. The lake is decomposed, the blue changes to red, and the haematein, set free again, begins to dissolve out. The slide is placed once more in tap water. When it is 'blued' again, further microscopical examination will show whether the blue colour is confined to the chromatin,

or whether some still remains in the ground cytoplasm. If so, the excess must be removed by further treatment with acid, and then a further blueing completes the process.

The most important of all dyes in cytology is 'iron haematoxylin', the lake with iron ammonium sulphate, $Fe_2(SO_4)_3 + (NH_4)_2SO_4 + 24H_2O$, which is bought as beautiful large pale-violet crystals, giving a yellow solution. (Mann (1902) made an error with the formula for iron alum.)

This method was announced by Benda at a meeting of the Berlin Physiological Society on 28 May, 1886. His method was the same as that now in use, except that he used 0·05% chromic acid to remove excess of stain. The modern method was introduced by M. Heidenhain in 1892, in a course of a study of the nucleus and centriole of the white-blood corpuscle of the salamander. Heidenhain gave fuller instructions in 1896, in a further study of the centriole and the cytoplasm surrounding it. Heidenhain himself used to stain for an unnecessarily long time. With this technique the mordanting is done first, then the staining, and then the extracting of the excess of stain. One puts the slide first in 2½% iron alum in water for one or two hours after Carnoy or Bouin, or for about twelve hours after Flemming or Altmann. One then rinses the slide in distilled water, and transfers it to the haematoxylin solution and leaves it there as long as it was in the mordant. The stain is made up as follows: Dissolve 1 gram of haematoxylin in 10 c.c. of alcohol: add 90 c.c. of distilled water. Keep this stock solution for a month or more to ripen. For use, dilute it with an equal volume of distilled water. Everything is stained intensely black, so that no structure whatever is visible. Rinse off the loose stain, and then put the slide back again in the mordant. It always surprises people to hear for the first time that the mordant removes the stain, but actually it is not very surprising, for the iron alum

molecules that are floating about free in the solution attract the haematein ones just as much as those that have previously been fixed in the tissue. The haematein comes out in clouds, first from the ground cytoplasm, then from the mitochondria (if present), then from the centrioles, then from the chromatin of interkinetic nuclei, and finally from chromosomes. The tissue may be completely destained in this way, but the cytologist is careful to watch the process from time to time under the microscope, and to stop it at exactly the right time, according to the structure which he is studying. The differentiation usually takes between two and fifteen minutes. If there is any difficulty in getting mitochondria to stain, it is legitimate to do the mordanting and staining (but not the differentiation) in the paraffin oven. If chromosomes are clumped and it is difficult to count them, one may follow Lee (see Gatenby and Cowdry, 1928) in mordanting for only two and a half minutes and staining for only four. Short times such as these give a blue stain instead of black. When one wishes to stop the differentiation, one has only to put the slide into running tap water for five minutes.

The advantages of iron haematoxylin are that nearly everything in the cell may be stained by it; the differentiation is easy to control; the stain is extremely sharp; higher power eyepieces may be effectively used than after other stains; photography is easy; and it is absolutely permanent. Nearly all our knowledge of chromosomes, except the earliest, has been gained by the use of iron haematoxylin.

When a mordant attaches itself to a tissue, exactly the same questions arise as to the method of attachment as arise in the direct attachment of stains: but no one denies that the attachment between the mordant and the dye is a chemical combination. Different mordants often give different colours with the same dye: the iron and the chromium lakes of

haematoxylin are black or blue-black, while the aluminium and copper lakes are blue. In industry haematoxylin is chiefly used for dyeing blacks on silk and wool, with iron and chromium mordants.

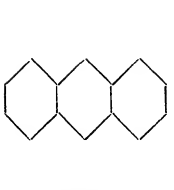
Madder

We have yet to discuss another vegetable dye which is used with a mordant. This is madder. Its use in cytology is restricted to one method for mitochondria, so it must be dealt with more shortly, despite its interest. Madder is the name of a genus of the Rubiaceae, the coffee and cinchona family. Common Madder, *Rubia tinctorum*, grows a few feet high. It is probably a native of southern Europe. Its roots contain a glucoside from which the glucose can be separated by acids, leaving the dye madder or alizarin. Madder is especially used in industry for dyeing Turkey Red, with aluminium as the mordant. The bones of animals which eat madder roots become red.

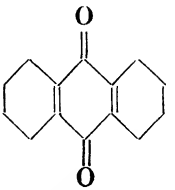
It was discovered in 1869 that alizarin could be prepared from anthracene. This was the first synthesis of a natural colouring matter. By 1903 natural madder had disappeared from British imports.

Anthracene is obtained from those portions of coal-tar which boil between 340° and 360° C. It forms laminae with a blue fluorescence. Oxidizing agents convert it into anthraquinone. Chromic acid may be used as oxidizer, the anthracene being dissolved in pure boiling acetic acid. The dihydroxy-derivative of anthraquinone is alizarin. The fact that the two hydroxyl groups are in the ortho-position is shown by the possibility of preparing alizarin from catechol. Alizarin forms bright orange-red needles. Alizarin S is the sodium sulphonate of alizarin, and this is the dye with which we are concerned. It is used for dyeing wool, chiefly closely woven goods, on account

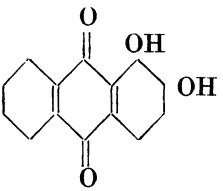
of its good penetration. The reader will note that



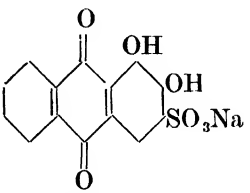
Anthracene



Anthraquinone



Alizarin



Alizarin S.

once again we are dealing with a quinonoid dye. Madder is an acid dye, giving splendid colours with basic mordants ; scarlet (Turkey Red) with aluminium, maroon with chromium, violet with iron, and orange with tin. When a piece of tissue is fixed in a fluid containing chromic acid (e.g. Flemming), and is subsequently mordanted on the slide with iron alum and stained with alizarin S, a yellowish to rose pink is produced. Nucleus and cytoplasm are both stained, the former more deeply.

Benda, the namer of mitochondria, introduced his technique for them, involving alizarin S, in 1901, and subsequently perfected it (1902, 1927). In my opinion this is the finest of all methods for mitochondria, but it has been much neglected of late, perhaps because Benda has himself stipulated some quite unnecessary and troublesome complications, such as fixing the sections on coverslips, dehydrating with filter paper, and treating with the very expensive Bergamot oil before xylol. I have made a special

study of this method, and I give it here exactly as I myself carry it out. The principle of the technique is as follows: (1) One mordants for alizarin staining with chromium in the fixative and iron alum on the slide. (2) One stains with alizarin S, which itself, as an acidic substance, mordants for the basic dye, crystal violet. (3) One stains with crystal violet, which makes everything violet. (4) One extracts the crystal violet with acetic acid. The mitochondria retain it longer than any other part, and they are sharply stained a blueish violet and show up well against the nucleus and cytoplasm, which are stained yellowish to rose pink with the alizarin.

First make up the necessary solutions.

(1) 4% iron alum.

(2) Alizarin S. Make a saturated stock solution in 90% alcohol. For use, add 1 c.c. of the stock solution to a slide jar of distilled water (about 80 c.c.). Make up afresh each time.

(3) Crystal violet, sat. sol. in 70% alcohol 25 c.c.
Alcohol, 70%, with the addition of 1% of conc. HCl . . . . 25 c.c.
Aniline, sat. sol. in water . . 50 c.c.

I much prefer this solution, which keeps well, to Benda's newer solution.

(4) 30% acetic acid.

Fix small pieces in Flemming with reduced acetic for four days to a week. They may be postchromed or not. Cut sections 5 μ or less. Bring them to water, and leave a day in iron alum. Rinse for a moment in a stream of distilled water, and leave for a day in alizarin S. Rinse as before. Dry the slide below and also above with a duster, as far as is possible without touching the sections. Place the slide on a tripod and flood it with crystal violet solution. Warm with a very small Bunsen flame until the fluid just starts to steam, and then stop warming at once. Care must be taken to prevent the fluid from catching fire, as a precipitate is formed if

this occurs, and the sections are unduly heated. Leave the slide for about five minutes to cool. Place it in a stream of running tap water to remove the excess of stain. Now transfer it to a slide-bottle of 30% acetic acid, which differentiates the violet stain very rapidly. For a first trial, twenty seconds in the acetic acid is usually a convenient time. Put the slide back in running water to stop differentiation. After washing (a few seconds suffice) examine the slide with a 4-mm. objective. Differentiation is complete when the crystal violet has been removed from the cytoplasm and nuclei, but left in the mitochondria. Sometimes it is necessary to leave a tinge of blue in the nuclei, for otherwise the stain will be too far removed from the mitochondria. In an ideal preparation the mitochondria are blueish violet, while the nuclei and cytoplasm are stained yellow to brown or rose with alizarin. The nuclei stand out, as they are much darker than the cytoplasm. If differentiation is not complete, return the slide to the acetic acid for five to ten seconds, wash again in running tap water, and once more examine under the microscope. This may have to be repeated many times before differentiation is carried out to the proper point. In cases where difficulty is experienced in differentiating to the right degree, I find it convenient to make a long ribbon of sections and attach it down the middle of the length of the slide. One very gradually immerses the slide in the acetic acid, in such a way that the section at one end of the slide is covered for two minutes, while that at the other end is only momentarily covered. One of the sections is almost sure to be correctly differentiated, though occasionally up to ten minutes' differentiation is required. Wash the slide for five minutes (or longer) in running tap water, to remove all traces of acidity. Dehydrate with acetone instead of alcohol, as crystal violet is almost instantaneously removed by alcohol. (See p. 117.) The finished sections often look well if

examined by electric light without the intervention of any screen.

This method has the advantages that the mitochondria are very sharply stained and show up clearly against their background, that nuclei and chromosomes are quite well stained, and that the preparations do not fade. It is ideal for work on spermatogenesis, for the chromosomes are sufficiently well preserved and stained for it to be possible to identify the stage of maturation of each cell. It may be used, though less well, after Altmann fixation, or after formaldehyde fixation followed by postchroming.

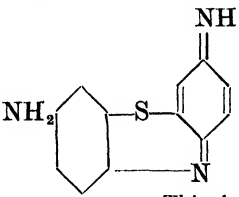
CHAPTER VII

STAINING (*continued*)

IN the last chapter we have considered the general principles of staining, by reference to the triphenyl dyes, and of mordanting, by reference to haematoxylin and madder. Let us now turn to those synthetic dyes which are not of the triphenyl type.

Thiazin Dyes

The thiazin dyes contain a quinonoid ring, linked to a non-quinonoid ring by a nitrogen atom and a sulphur atom. The prefix 'thi-' of course refers to the sulphur atom. Thionin, a blue dye with a reddish tinge, is an example. I have modified the way of writing the formula slightly, to bring out the quinonoid structure clearly. The two amino groups make thionin strongly basic. It will be noticed that it is a chloride.



Thionin

In industry it is used for dyeing cotton, tannin being used as a mordant. In cytology it is used for staining chromatin, no mordant being required to attach this basic substance to the acidic nucleoproteins. It is so strongly basic that at first the cytoplasm is untouched, even in neutral solutions. After fixation in Flemming,

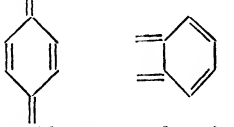
sections may be stained for three to fifteen minutes in a 1% solution, rinsed with distilled water, and dehydrated at the usual speed. (See p. 117.) A minute's treatment with very dilute hydrochloric acid (10 drops in a staining jar of distilled water) removes all blue except from chromosomes. Overstained sections may be destained by dipping in dilute acid or by prolonged soaking in neutral alcohol. After Flemming, only the properly fixed cells are properly stained. The nuclei of the outermost cells and of the cells of the interior of the piece may be scarcely touched with blue, while the well-fixed cells lying between are sharply stained. I have found orange G. a good counter-stain. (See p. 111.) The only disadvantage of thionin is a tendency to fade.

Thionin stains chromatin blue, but mucin and the ground substance of cartilage purple. The phenomenon of the staining of different tissue elements in different colours by the same dye is called metachromasy.

Another thiazin dye is toluidin blue, which has been much used for staining chromatin in Kull's technique for mitochondria. It has unfortunately a tendency to stain other parts as well in this technique. Methylene blue is another basic thiazin dye.

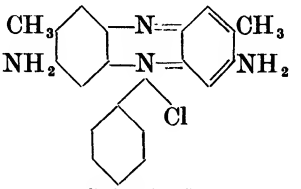
Azin Dyes

It might have appeared rational to take the azin dyes before the thiazin, but the azin dyes introduce a new form of the quinonoid linkage, so I have deferred them. In azin dyes the quinone is an orthoquinone, instead of a paraquinone, which it has been in all the other dyes we have studied. The ortho-



Paraquinonoid ring Orthoquinonoid ring

quinonoid ring, like the other, has two double bonds projecting from it, but they are in the ortho-position to one another. In azin dyes there are an ortho-quinonoid and a benzene ring, linked together by two nitrogen atoms. In the safranins one of these nitrogen atoms has another benzene ring attached to



Safranin O.

it in addition. Safranin O is shown above. Actually it is a mixture of the substance shown with another differing only in having a methyl group attached to the lower ring. It will be noticed that safranin O is a chloride and a strongly basic dye. It is a splendid dye for chromatin and particularly for chromosomes. It stains well after Flemming, but poorly after Bouin. It is said that the chromic acid helps it to stain, but I am inclined to think that the osmium is important, for it scarcely stains in the interior of the piece, which is fixed by the chromic but not by the osmium. The cells lying on the surface, which are ' overfixed ' by the osmium, are, however, not strongly stained. It is the well-fixed cells, lying just below the surface, that take the stain strongly.

One may keep a 3% solution in 50% alcohol as a stock solution. For use this may be diluted with four times its volume of 50% alcohol. Aniline is often used as an accentuator, but this is not necessary, and tends to make the solution deteriorate.

After Flemming, stain in safranin for twenty-four hours. Wash off the excess of stain in a stream of distilled water. Differentiate in 70% alcohol until scarcely any tinge of red is left in the cytoplasm.

This takes between half a minute and ten minutes. Then dehydrate at the standard speed. (See p. 117.) The last traces of red disappear from the cytoplasm during dehydration.

If it is desired to show chromosomes only, and not interkinetic nuclei or early prophases, one may differentiate in 1% acetic acid in 70% alcohol. After staining, wash off the excess of stain in a stream of distilled water, and differentiate in acid alcohol for five seconds. Wash again in a stream of distilled water, and examine under the microscope. Repeat this process again and again, if necessary, until the stain is almost confined to the chromosomes, interkinetic nuclei being practically destained. Then dehydrate at the standard speed.

The use of light green as a counterstain for safranin was introduced by Benda (1891). He gives no very precise instructions. I find it best to proceed as follows. Sections of Flemming material, 5 μ or less thick, are stained for twenty-four hours in safranin. An open slide bottle of $\frac{1}{4}$% light green in 70% alcohol and an open slide bottle of 70% alcohol are placed beside the microscope. The slide is rinsed quickly in a stream of distilled water, dipped for an instant into the light green, dipped into the alcohol, and examined quickly under the microscope (16 mm. or 4 mm. objective, according to the size of the cells). The object is to remove all trace of red from the cytoplasm, substituting a very pale green, while the chromatin should be red. If any red remains in the cytoplasm, the slide is returned to the light green for an instant and the whole process repeated, many times if necessary, until the desired result is obtained. Usually about three dips in the light green are required, and differentiation is finished forty-five seconds after the first dip. The slide is then dipped in 90% alcohol, placed in absolute alcohol, and carried through as usual. (See p. 117.) There is a great danger of differentiating too far, and extracting the safranin from the chromatin. If this

happens, one must stain for twenty-four hours in safranin again. A still greater danger is that one may be so frightened of over-differentiating that one does not carry it far enough, and the cytoplasm is a dirty mixture of red and green. It is not an easy technique, but successful preparations are excellent, as the red shows up brilliantly against the very pale green.

For chromosome work safranin has probably been used more than any other dye except haematoxylin. It has certain advantages. The chromosomes remain transparent, which helps in counting them when one lies behind another. Also karysomes and chromosomes are stained red, while plasmosomes are stained green by Benda's technique. This is a great help, especially in spermatogenesis. Iron haematoxylin, of course, does not distinguish them. Like haematoxylin, safranin is permanent.

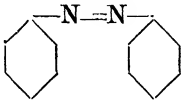
Other basic azin dyes are 'neutral' red and Janus green B. The latter is both an azin and an azo dye. These are both intravitam stains, the former for neutral red vacuoles, the latter for mitochondria. Mauve, the first aniline dye to be invented, is an azin dye. Probably it was the first aniline dye used in cytology. (See Conn, 1925.)

Azo Dyes

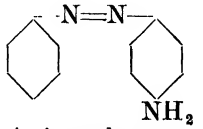
All the dyes we have dealt with so far, both synthetic and vegetable, depend on a quinonoid linkage somewhere in the molecule for the fact that they are coloured substances. Many other dyes have no quinonoid linkage, but rely for colour upon quite different arrangements of the molecule. It must be remarked that no one knows exactly why certain arrangements give colour, but of the fact there is no doubt. In diazo dyes (generally called azo dyes), the linkage which gives colour is $-N=N-$. 'Diazo' means two-nitrogen.

Diazo compounds are formed by the action of nitrous acid (or nitrites) on an amino-derivative of

benzene. One nitrogen atom comes into the molecule from the nitrous acid, the other from the amino group, and they link together as shown above. Such substances are coloured, but they are not necessarily dyes. To be able to attach themselves to tissues or fabrics, they must have an OH or NH_2 or other such group. (The original NH_2 group of the amino-benzene no longer exists when the diazo-linkage has taken place.) Azobenzene is a coloured substance: aminoazobenzene is a dye.



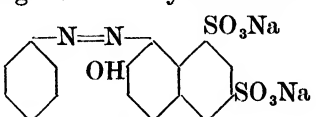
Azobenzene



Aminoazobenzene

Let us produce a brightly coloured diazo compound, closely allied to aminoazobenzene. Dissolve 1 gram of sodium nitrite in half a litre of distilled water, and 6 grams of aniline hydrochloride in another half-litre. Mix the two solutions. The fluid, previously colourless, becomes bright yellow in a few minutes. Sufficient aniline was present not only to provide one of the nitrogen atoms for the diazo-linkage, but also to give the NH_2 group required for the formation of the dye.

There are thousands of different azo dyes, acid and basic, but we shall only consider one, orange G. The letter G. (gelb) refers to the yellowish colour given by the particular positions of the sulphonic and hydroxyl groups. Other arrangements give a reddish orange. Orange G. is chiefly used in industry for the



Orange G.

production of compound shades, with other dyes, on

wool. It is very fast to light. In cytology it is best to use it as a saturated solution in absolute alcohol, and simply to pass one's slides through this instead of the first plain absolute alcohol when dehydrating previous to mounting. (See p. 117.) One may stain for two minutes. Longer times are not harmful, as it does not overstain. The formula shows this to be an acid dye, and it is best used for staining the cytoplasm when the chromatin has been stained blue or black. It has the advantages over eosin as a routine counter-stain that it gives greater transparency, is more selective, and does not overstain. For this reason eosin has not been considered in this book.

Other azo dyes are sudan III and IV. These peculiar dyes present also a quinonoid linkage, and are neither basic nor acidic. They are soluble in fat.

Nitro Dyes

In nitro dyes colour is given neither by a quinonoid nor an azo linkage, but by the presence of a nitro group, NO_2. Picric acid is an example. We have already discussed the chemistry of it, since it is a fixative as well as a dye. It is pale yellow in colour. Its special value in cytology is as a counter-stain to acid fuchsin in Altmann's method for mitochondria (1890). By this method mitochondria are stained red by acid fuchsin, and everything else in the cell yellow with picric acid. I adopt Metzner's modification of Altmann's technique, as given by Benda (1927), with small modifications. The idea of the technique is to stain everything in the cell red with acid fuchsin, and then to extract the red from everything except the mitochondria, replacing it with the pale yellow of the picric acid.

First make up the necessary solutions.

(1) Acid fuchsin, 12% in aniline water. To make aniline water, shake up about 5 c.c. of aniline with 100 c.c. of water and filter. Altmann recommended a 20% solution of acid fuchsin, but this amount will

not dissolve. Gatenby (1928) says that he can only get it to dissolve at 5–7%. I find that it dissolves at 12%, but not at much higher concentrations. Keep the acid fuchsin solution in a bottle.

(2) Stronger picric acid. Twenty parts of a saturated solution of picric acid in absolute alcohol and 80 parts of 20% alcohol. Very weak alcohol is taken. If stronger is used, the fluid may take fire at a later stage, if warming is necessary.

(3) Weaker picric acid. The same as the last, but in the proportion of only 10 parts of the picric solution to 90 of the 20% alcohol.

The picric solutions are to be used in staining jars.

Treat Altmann or any postchromed tissue as follows. Cut sections at 5 μ or preferably less. Bring them to water. Dry the bottom of the slide with a duster, and also the top of it except where the sections lie. Place the slide on a tripod. Flood it with acid fuchsin solution. It is best to have a lot of stain on the slide ; otherwise evaporation causes solid stain to be precipitated round the edges of the slide, which is troublesome to remove. Heat the slide with a Bunsen burner. Do not hold the flame steady below the slide, but move it about, so that it is only directly under the slide for part of the time. If it is held steadily below the slide, the fluid may boil and the section be spoilt. Also the slide may crack. Heat it until it steams, and continue heating so as to keep it steaming for one minute by the clock. Then leave it for about five minutes to cool. Place a rubber glove on whichever hand is customarily used for moving slides during microscopical examination. Lift the slide over a sink and pour away as much of the stain as will run off. Wash rapidly with a stream of *distilled* water. Place at once in the stronger picric solution. The red stain begins to be extracted at once. After half a minute quickly remove the slide and put it as it is on the stage of the microscope. Examine very rapidly with a 4-mm. objective.

Differentiation may already be nearly complete. It is complete when the nuclei are distinctly yellow and the cytoplasm very pale yellow, while the mitochondria are still bright red. If it is nearly complete, transfer the slide to the weaker picric solution, and examine it as before about every half-minute until differentiation is complete. If at the first examination differentiation is not nearly complete, replace the slide in the stronger picric solution and keep examining it every half-minute until it is nearly complete. It may then be transferred to the weaker solution until quite complete. One must of course be careful not to differentiate too far, so that the dye becomes extracted from the mitochondria. Occasionally the acid fuchsin does not leave the cytoplasm and nuclei even after a long time in the strong solution. If this happens, dry the bottom of the slide, put it on the tripod, flood it with the weaker solution, and warm it with a Bunsen until it just begins to steam. Then stop heating it immediately. As soon as it is cool enough, replace it in the jar of weaker picric and continue as before.

Immediately differentiation is complete, wash the slide rapidly in a stream of distilled water, dip it quickly in 70% and 90% alcohol, and then leave it the standard two minutes in absolute alcohol, before passing as usual into xylene. (See p. 117.)

The advantages of this technique are the quickness, certainty and simplicity of it. Its disadvantages are that (1) the red of the mitochondria does not show up so clearly as it would if the cytoplasm were of a complementary colour ; (2) the red colour gradually fades ; (3) chromatin does not show up clearly, as it is of the same colour (yellow) as the cytoplasm, and it is therefore impossible, in spermatogenesis work, to determine the exact stage at which the cell has arrived ; and (4) it is occasionally impossible to get the nuclei yellow without also destaining the mitochondria.

This method seems on the whole preferable to the more difficult Bensley (1911) technique, in which methyl green is used to differentiate the acid fuchsin, though this sometimes gives excellent results. It is almost certainly preferable to the Kull (1913) technique, in which the nucleus is stained blue by toluidin blue and the cytoplasm yellow with aurantia. The blue often stains diffusely, and the aurantia destains the acid fuchsin too rapidly for accurate differentiation. Acidification of the blue stain should improve this technique.

Aurantia, the yellow dye just mentioned, is the only nitro dye except picric acid that is at all commonly used in cytology.

Carmine

Despite its great interest, the animal dye carmine has been left to the last and only a paragraph can be devoted to it, for nowadays it is important in micro-anatomy rather than in cytology. In the early days of staining it was much used in cytology, and it is still used in Best's method (1906) for the demonstration of glycogen. It occurs in the female Homopteran Coccus cacti. This scale insect lives upon the cactus, *Opuntia coccinellifera*, which grows in tropical America and has been introduced into the Canary Islands. The dried females constitute cochineal, from which the red dye carmine may be extracted. From carmine a complex organic compound, carminic acid, may be separated. Carminic acid may be used as a dye if mixed with aluminium and calcium salts as mordants. Carmine itself may be used without added mordants, since it conveniently contains both aluminium and calcium. Since it is only slightly soluble in water, it is dissolved in borax solution. Carmine and carminic acid may be differentiated with acid alcohol. Differentiation may proceed until the cytoplasm is colourless and the nuclei still red. The chief use of carmine depends upon its great power of pene-

tration, which makes it possible to stain whole blocks of tissue, or complete small animals, instead of sections. When the stain has been differentiated and the preparation mounted in balsam without sectioning, some of the organs show up clearly because they contain more nuclei than others, and therefore retain more of the stain. Carmine is quite permanent.

CHAPTER VIII

MOUNTING

THE stained slide has to be dehydrated, for any water in it would prevent its impregnation with the mounting medium. The best way of dehydrating is generally to leave the slide about a quarter of a minute in 70% alcohol, about the same length of time in 90% alcohol, and then to dip it into one jar of absolute alcohol and leave it two minutes in a second jar of absolute alcohol. The dip in the first jar prevents 90% alcohol from being carried over and thus preventing the final dehydration. With dyes like haematoxylin, which are not extracted by alcohol, the times given may be increased indefinitely, but no advantage accrues. Sometimes it is necessary to pass more rapidly through 70% and 90% alcohol, in order to avoid extraction of a dye. Crystal violet is extracted very rapidly by alcohol. When using Benda's technique for mitochondria (p. 102), I therefore dry the slide with a duster except just round the sections, and dehydrate for one minute in pure acetone. (Benda dehydrated with filter paper and Bergamot oil.) Acetone, $(CH_3)_2CO$, is a colourless liquid which mixes with water in all proportions. It distils over when dry metallic acetates are heated.

A go-between is now required to enable the tissue to pass from alcohol or acetone to the mounting medium, Canada balsam. For this purpose one requires a fluid which mixes in all proportions with alcohol, and also dissolves Canada balsam. Most of the essential oils satisfy these conditions, but they are

often messy and expensive and have a tendency to extract dyes. Benzene or chloroform could be used, but they evaporate so quickly that the application of the coverslip is difficult. The ideal substance is xylene (see p. 79), which satisfies all the conditions. One dips the slide in a jar of xylene, to wash off the excess of alcohol, and then leaves the slide for two minutes in another jar of clean xylene, to extract the alcohol from the section. The period in xylene may be prolonged indefinitely without damage or advantage.

The section is now ready to be mounted. The mounting medium is a sticky solution of Canada balsam in xylene.

Canada balsam is the oleoresin which is collected from blisters which occur on the bark of the Balsam Fir, *Abies balsamea* (Dallimore and Jackson, 1923). This tree, 25 to 60 feet high and 2½ to 5 feet in girth at the base, is a native of North America, where it has a wider distribution than any other species of the genus. It ranges from Newfoundland to Lake Superior, and from low-lying swampy ground to 5,000 feet. The genus Abies, or Silver Firs, is distinguished from the genus Picea, or Spruces, by having the cones erect instead of pendulous and by the fallen leaves leaving no peglike projections on the twigs. *A. balsamea* differs from most of the Silver Firs in having the leaves in two distinct rows, one on each side of the twig, in having resin canals along the middle of the leaves, instead of along the sides, and in having resinous winter buds. The wood is useless for most purposes, but a little is pulped for paper. The Balsam Fir does not succeed well in this country, because it starts growing directly warm weather comes, and the growing shoots are affected by subsequent frosts. Fairly large trees have been grown in Scotland. The drawing below shows a twig with winter buds from a small tree in my possession. The oleoresin is not only used in microscopy, but also in varnishes.

Oleoresins are essential oils in which resins are dis-

solved. Resins are themselves oxidation products of essential oils. 24% of Canada balsam is essential oil. The rest consists mostly of a resin soluble in both alcohol and xylene, but partly of a resin which is not soluble in alcohol but which is soluble in xylene. Alcohol is thus only a partial solvent for balsam, while xylene dissolves it wholly. The name 'balsam' is really incorrect, for it is properly restricted to oleo-resins containing benzoic or cinnamic acid, and hence possessing curative properties.

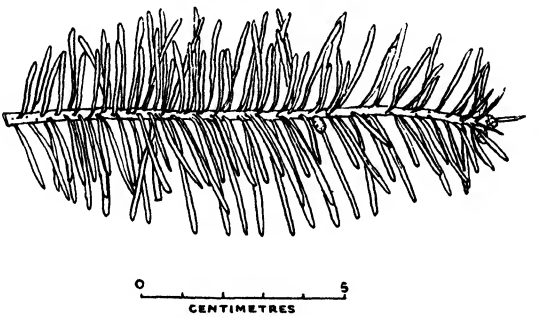


FIG. 3.—A Twig of *Abies balsamea*
(*Drawn by* Zita Baker)

Canada balsam is a very thick transparent pale-yellow fluid. Its advantages as a mounting medium are (1) that it is transparent and almost colourless in thin layers, (2) that it scarcely darkens with time, (3) that it dries hard and sticks firmly to glass when the essential oil is driven off by heat, (4) that its refractive index (1·541) and dispersion are close to those of glass. Its only disadvantage is that it has an acid reaction, which sometimes causes dyes to fade.

To make Canada balsam sufficiently fluid to form a thin layer between slide and coverslip, it should be diluted with xylene. The amount of xylene should be sufficient to make a fluid which will drop off a glass rod.

Before mounting a slide, dip a thin coverslip in acid alcohol and dry it thoroughly, handling it by the edges only. Prop it up so that it does not get dirty by contact with the bench. Remove the slide from the xylene. Quickly dry the bottom and ends of it with a dry duster and lay it on the bench. Make a streak of diluted Canada balsam along the middle of the coverslip. Lower the coverslip slowly on to the slide with a needle before the xylene has had time to evaporate off the section. Care should be taken not to breathe on the slide during mounting, as water vapour would naturally be harmful.

Put the slide on the hot plate to dry. In a quarter of an hour it will be dry enough for examination, if too much balsam was not used. For permanent preservation one should dry the slide on the hot plate for two days, so as to drive off nearly all the xylene and essential oil, and leave the hard resin behind to hold the coverslip firmly in place.

When at last we have finished our preparation, our labours will be wasted unless we really understand how our microscope works and how to use it, a subject which is often neglected. Fortunately there are excellent inexpensive books on the subject (Marshall and Griffith, 1929 ?, and Martin and Johnson, 1931).

We have followed the tissue from the living animal to Canada balsam. It is my hope that we have some understanding of what we have done.

APPENDIX

Suggested Material and Techniques for Beginners

Chromosomes. Testis of newt in late July or early August. Fix in Bouin and stain in iron haematoxylin (pp. 65 and 99), or fix in Flemming and stain in safranin and light green (pp. 67 and 109).

Mitochondria. Liver of newt or kidney of cavy. Fix in 10% formaldehyde for a day; postchrome; stain in iron haematoxylin (pp. 26, 48 and 99).

Liver or small intestine of cavy. Fix in Altmann. (It is suggested that Mr. Thomas's and my modification may be preferable to the original formula.) Stain with acid fuchsin and picric acid (pp. 69 and 112).

Testis of cavy or intestine of salamander. Fix in Flemming with reduced acetic; do not postchrome; stain by Benda's method. The mitochondria in the late spermatids of the cavy are very easy to stain, but some practice is necessary for those of the primary spermatocytes (pp. 67 and 102).

Golgi bodies. Epididymis or dorsal root ganglia of cavy. Fix in Mann. Postosmify (pp. 71 and 79).

Fallopian tube or dorsal root ganglia of youngish cavy. Da Fano's method (pp. 73 and 79).

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